

**Drug resistant malaria in Papua New Guinea and molecular  
monitoring of parasite resistance**

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Prof. Dr. Hans-Peter Hauri

Dekan

*To my beloved grandmother,*

*Josephine Marfurt*



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**Abbreviations**

<b>ACT</b>	Artemisinin-based combination therapy
<b>AQ</b>	Amodiaquine
<b>ATQ</b>	Atovaquone
<b>bp</b>	Base pairs
<b>CG</b>	Cycloguanil
<b>CQ</b>	Chloroquine
<b>CQR</b>	Chloroquine resistance
<b>CYT <i>bc</i><sub>1</sub></b>	Cytochrome <i>bc</i> <sub>1</sub> complex
<b>DAP</b>	Dapsone
<b>DDT</b>	Dichlorodiphenyltrichloroethane
<b>DHFR</b>	Dihydrofolate reductase
<b>DHPS</b>	Dihydropteroate synthase
<b>DNA</b>	Desoxyribonucleic acid
<b>DOX</b>	Doxycycline
<b>DV</b>	Digestive vacuole
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b><i>glurp</i></b>	Glutamate-rich protein
<b>HAL</b>	Halofantrine
<b>HC</b>	Health centre
<b>IPT</b>	Intermittent preventive treatment
<b>IRS</b>	Indoor residual spraying
<b>ITN</b>	Insecticide treated bednet
<b>LUM</b>	Lumefantrine
<b>MALDI-TOF</b>	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
<b>MDA</b>	Mass drug administration
<b>MEF</b>	Mefloquine
<b>MOI</b>	Multiplicity of infection
<b><i>msp1</i></b>	merozoite surface protein 1
<b><i>msp2</i></b>	merozoite surface protein 2
<b>NACT</b>	Non-artemisinin-based combination therapy
<b><i>pABA</i></b>	Para-aminobenzoic acid

<b>PCR</b>	Polymerase chain reaction
<b><i>PfATPase6</i></b>	<i>P. falciparum</i> ATPase6
<b><i>pfcr1</i></b>	<i>Plasmodium falciparum</i> chloroquine resistance transporter
<b><i>pfdhfr</i></b>	<i>Plasmodium falciparum</i> dihydrofolate reductase
<b><i>pfdhps</i></b>	<i>Plasmodium falciparum</i> dihydropteroate synthase
<b><i>pfmdr1</i></b>	<i>Plasmodium falciparum</i> multidrug resistance gene 1
<b>PG</b>	Proguanil
<b>Pgh1</b>	P-glycoprotein homolog 1
<b>PNG</b>	Papua New Guinea
<b>PNGIMR</b>	PNG Institute of Medical Research
<b>PRIM</b>	Primaquine
<b><i>pvdhfr</i></b>	<i>P. vivax</i> dihydrofolate reductase
<b><i>pvdhps</i></b>	<i>P. vivax</i> dihydropteroate synthase
<b><i>pvmr1</i></b>	<i>P. vivax</i> multidrug resistance gene 1
<b>PYR</b>	Pyrimethamine
<b>QUIN</b>	Quinine
<b>RBM</b>	Roll Back Malaria
<b>RFLP</b>	Restriction fragment length polymorphism
<b>SDX</b>	Sulphadoxine
<b>SERCA</b>	Sarcoendoplasmic reticulum Ca <sup>2+</sup> -ATPase
<b>SNP</b>	Single nucleotide polymorphism
<b>SOP</b>	Standard operating procedure
<b>SP</b>	Sulphadoxine-pyrimethamine
<b>SPR</b>	Sulphadoxine-pyrimethamine resistance
<b>TET</b>	Tetracycline
<b>UTL</b>	Useful therapeutic life
<b>VP</b>	Verapamil
<b>WHO</b>	World Health Organization

## Summary

Malaria is a serious global health problem and in the absence of an effective vaccine, access to safe and effective treatment still remains the mainstay in the control of the disease. However, the efficacy of this control strategy is hampered by the emergence and spread of drug resistant malaria which may lead to excess of mortality. One of the greatest challenges for health authorities of malaria endemic countries is thus to decide on when and how antimalarial drug policy should be changed, so that most of the patients will fully recover from the disease and will be cleared from parasites.

The current 'gold standard' for the assessment of antimalarial resistance is the estimation of *in vivo* drug efficacy, whereas *in vitro* drug sensitivity tests and the analysis of molecular resistance markers in the parasite serve as complementary tools.

In the present study, we assessed the relevance of a new appraisal approach for malaria resistance: community-based cross-sectional surveys versus clinical malaria studies, and the usefulness of a new molecular technology for the identification of molecular markers in different parasite genes. The frequencies of single nucleotide polymorphisms (SNPs) in given resistance marker genes, as well as genotype patterns were analyzed in clinical samples and their role in predicting *in vivo* treatment response was investigated. Furthermore, community drug resistance profiles were correlated with the incidence risk of clinical treatment failure in order to evaluate the relevance and usefulness of such a novel approach in the management of drug use.

In Papua New Guinea (PNG), the 4-aminoquinoline drugs amodiaquine (AQ) and chloroquine (CQ) have been first-line treatment against uncomplicated malaria until the late 1990s. At the same time, resistance of *Plasmodium falciparum* and *P. vivax* to these drugs had reached unacceptably high levels and health authorities were prompted to revise antimalarial treatment policy in 1997. First efficacy trials with the combination of AQ or CQ plus SP conducted between 1998 and 1999 showed good efficacy against falciparum and vivax malaria and the PNG Department of Health chose these combination regimens to replace the monotherapy with AQ or CQ as the standard first-line treatment against uncomplicated malaria in 2000.

The *in vivo* studies we conducted between 2003 and 2005 were the first ones to assess the therapeutic efficacy of the newly introduced combination regimen against *P. falciparum* and *P. vivax* malaria using the revised WHO standard protocol. In our studies conducted in three different areas over the period of three consecutive years, we observed PCR-corrected treatment failure rates up to 28% for *P. falciparum* and 12% for *P. vivax* malaria.

Regarding former drug history in PNG (i.e., long lasting 4-aminoquinoline use and sporadic use of SP as mass chemoprophylaxis or partner drug with quinine for second-line treatment), we found a genetic background in the parasite population that is associated with high CQ as well as moderate pyrimethamine resistance. We also observed the emergence of mutations concordant with a sulphadoxine resistant phenotype, indicating that the efficacy of the sulpha component is already compromised. Further results that identified key *pf dhps* mutations to be most relevant in predicting treatment failure with the current first-line regimen corroborated our findings that AQ and CQ as inefficacious partner drugs of SP in the new standard treatment were not able to curb both, the progression of pyrimethamine resistance as well as the emergence of sulphadoxine resistance in PNG.

We have shown that our community-based molecular monitoring approach was feasible in PNG and that molecular monitoring of parasite resistance can indeed be a valuable supplementary tool in malaria resistance surveillance. However, our data also clearly highlighted several drawbacks of the presently applied methods for the assessment of resistance, the most important being the lack of standardised methods that are applicable in different epidemiological settings. In addition, our data indicate that currently suggested public health models for the molecular monitoring of parasite resistance are not suitable for universal application in settings which are different with regard to several factors such as malaria endemicity, transmission intensity and drug use patterns.

To summarize, decreasing *in vivo* efficacy of the current first-line regimen in PNG and the molecular drug resistance profile of the parasite population consistent with a CQ and SP resistant phenotype strongly indicate that a policy change to artemisinin-based combination therapy (ACT) has to be considered in the near future. We have shown that a careful baseline evaluation of the molecular resistance background is needed for the identification of the most relevant molecular markers for longitudinal monitoring in a given area. The novel DNA microarray-based method which allows the parallel analysis of multiple drug resistance-associated SNPs has been proven to be a valuable tool to assess the usefulness of each known molecular marker in a particular region with specific drug use. Moreover, the new technology enabled the assessment of molecular markers on an epidemiological scale and hence opened the avenue for the investigation of a more comprehensive community-based monitoring programme.

To conclude, the novel technical tool for the assessment of molecular markers of parasite resistance presented in the current study is cheap, easy to use, and applicable in laboratories

with limited infrastructure. Moreover, the technology is highly versatile and allows rapid adaptation to specific monitoring needs, the most important at the moment being the close monitoring of resistance to the highly effective artemisinin derivatives and potential partner drugs in ACTs. Though molecular markers have been proven to be useful as an early warning system, their usefulness in predicting treatment response and the progression of resistance is still limited. Hence, currently suggested public health models based on molecular data will have to include additional parameters for important determinants of parasite resistance and to be evaluated in varying epidemiological settings before molecular methods may eventually replace *in vivo* efficacy studies for the surveillance of resistance.

## Zusammenfassung

Malaria ist nach wie vor eine Tropenkrankheit mit immenser gesundheitspolitischer Tragweite. Da ein wirksamer Impfstoff in absehbarer Zeit nicht zur Verfügung stehen wird, ist die korrekte Diagnose der Krankheit und eine erfolgreiche Behandlung immer noch die wichtigste Kontrollstrategie gegen diese Infektionskrankheit. Die Strategie wurde aber in den letzten Jahren durch das Auftreten und die Verbreitung medikamenten-resistenter Parasiten stark beeinträchtigt und könnte in den nächsten Jahren die durch Malaria bedingte Morbidität und Mortalität erhöhen. Daher ist die Entscheidung, wann und wie die nationale Behandlungsstrategie gegen Malaria geändert werden soll, eine der grössten Herausforderungen für die Gesundheitsbehörden in Malaria-endemischen Gebieten.

Der Goldstandard zur Bestimmung der Medikamentenresistenz ist immer noch die Durchführung von klinischen Studien zur Erfassung der Wirksamkeit eines Präparats. Laborverfahren zur Bestimmung der *in vitro*-Resistenz gegen einzelne Wirkstoffe in Parasitenkulturen oder molekularer Resistenzmarker im Parasiten werden heute als zusätzliche Methoden eingesetzt.

In der vorliegenden Arbeit wurden zwei neue Ansätze zur Erfassung der Medikamentenresistenz untersucht. Zum einen wurden klinische Studien zur Bestimmung der Wirksamkeit des gegenwärtigen Behandlungsstandards in Papua Neuguinea (PNG) an mehreren Gesundheitszentren durchgeführt und von Querschnittsstudien in den Dorfgemeinschaften der entsprechenden Einzugsgebiete begleitet. Zum anderen wurde eine neue molekulare Methode zur Bestimmung von Punktmutationen in verschiedenen Parasitengenomen entwickelt und getestet. Hiermit wurde die Häufigkeit einzelner Resistenzmarker sowie auch die Muster unterschiedlicher Marker in klinischen Proben bestimmt, um diese Marker potentiell zur Vorhersage des Behandlungsergebnisses zu verwenden. Gleichzeitig wurde das Muster der Resistenzmarker in Parasiten in Proben der jeweiligen Querschnittsstudien bestimmt. Die verschiedenen Resistenzprofile der verschiedenen Regionen wurden mit der Inzidenz von Behandlungsergebnissen an den jeweiligen Gesundheitszentren verglichen. Hierbei war es Hauptziel, die Relevanz und Nützlichkeit eines solchen Ansatzes (Querschnittsstudien zur Bestimmung des molekularen Resistenzmusters in der Parasitenpopulation) zur Überwachung der Resistenzsituation abzuschätzen. Zusätzlich sollte beurteilt werden, inwiefern ein solcher Ansatz bei der Erstellung von Behandlungsstrategien hilfreich sein könnte.



In PNG wurden bis Ende der 90iger Jahre zur Behandlung klinisch unkomplizierter Malaria fast ausschliesslich Medikamente der 4-Aminoquinolin-Gruppe, Amodiaquin (AQ) und Chloroquin (CQ), verwendet. Gleichzeitig entwickelte sich Resistenz dagegen in *Plasmodium falciparum* und *P. vivax*. Nachdem die Resistenz ein nicht mehr akzeptables Ausmass angenommen hatte, waren die Gesundheitsbehörden in PNG 1997 schliesslich gezwungen, die Behandlungsstrategie gegen Malaria zu revidieren. Nachdem erste klinische Studien mit der Kombinationstherapie AQ oder CQ plus Sulfadoxin-Pyrimethamin (SP) zwischen 1998 und 1999 gute Wirksamkeit gegen falciparum- und vivax-Malaria gezeigt hatten, wurde diese Kombinationstherapie dann schliesslich im Jahr 2000 vom Gesundheitsdepartement offiziell zum neuen Behandlungsstandard erklärt.

Die hier vorgelegten klinischen Studien waren die ersten, welche die Effizienz der neuen Kombinationstherapie unter Berücksichtigung des neu revidierten Protokolls der Weltgesundheitsorganisation (WHO) bestimmt haben. In diesen Studien, die zwischen 2003 und 2005 in drei verschiedenen Regionen PNGs durchgeführt wurden, wurden Behandlungsmisserfolgsraten von bis zu 28% für *P. falciparum* und 12% für *P. vivax* gemessen.

Bedingt durch die bisherige Behandlungsstrategie in PNG (langanhaltender Gebrauch von 4-Aminoquinolinen und sporadischer Einsatz von SP während Massenbehandlungskampagnen) wurde auf molekularer Ebene ein hoher Grad an CQ-Resistenz und das Vorkommen von Pyrimethamin-Resistenz beobachtet. Gleichzeitig belegen die vorliegenden Daten das ansteigende Auftreten genetischer Parasitenresistenz gegen Sulfadoxin. Mutationen im *P. falciparum dhps*-Gen, welches hauptverantwortlich für die Sulfadoxinresistenz ist, waren auch die Marker mit dem besten Vorhersagewert für Behandlungsmisserfolg. Diese Daten zeigen, dass die pharmakologische Wirkung von AQ/CQ in der jetzigen Kombination nicht genügend war, um sowohl das Ausbreiten der Resistenz gegen Pyrimethamin, als auch das Entstehen der Resistenz gegen Sulfadoxin zu verhindern.

Es konnte auch gezeigt werden, dass die Erstellung eines genetischen Resistenzprofils der Parasiten in Querschnittsstudien in PNG erfolgreich eingesetzt werden kann und dass molekulare Marker wichtige Hinweise zur Wirksamkeit verschiedener Medikamente geben können. Die hier vorliegende Arbeit verdeutlicht allerdings auch die Probleme der gängigen Methoden zur Bestimmung der Resistenz, wie zum Beispiel das Fehlen von standardisierten Protokollen, die in Regionen mit unterschiedlicher Malariaepidemiologie gleichermassen angewendet werden können. Bisher vorgeschlagene Modelle zur molekularen Überwachung

von Medikamentenresistenz scheinen zudem nicht universell anwendbar zu sein. Dies ist möglicherweise auf Unterschiede in wichtigen epidemiologischen Eigenschaften wie Krankheitsendemizität, Übertragungsintensität, oder Gebrauch von Medikamenten zurück zu führen.

Zusammenfassend haben die klinischen und molekularen Resultate dieser Arbeit gezeigt, dass die Effizienz der heutigen Behandlungsstrategie in PNG sehr wahrscheinlich nur noch von kurzer Dauer sein wird und dass ein Wechsel zu einer auf Artemisinin-Derivaten basierenden Kombinationstherapie in Betracht gezogen werden muss.

Die Studie zeigte, dass eine umfassende Bestimmung der molekularen Marker und deren Einfluss auf den Behandlungserfolg wichtig ist, um geeignete Marker für die longitudinale Resistenzüberwachung zu identifizieren. Die hier vorgestellte DNA-Mikroarray Technologie zur Bestimmung von mehreren Punktmutationen in verschiedenen resistenz-assoziierten Genen hat sich als ideales Werkzeug für diesen Zweck erwiesen. Wichtige Kriterien, zum Beispiel einfache Handhabung und tiefe Kosten, machen die Methode praktikabel für Laboratorien mit beschränkten Mitteln. Sie ermöglicht einen grossen Probendurchsatz und ist daher für epidemiologische Studien geeignet. Als wichtiger Punkt sei die hohe Flexibilität des Systems erwähnt, da das Einschliessen verschiedenster Punktmutationen eine Überwachung der Resistenzdynamik von *P. falciparum* gegen bereits verwendete und/oder zurückgezogene, als auch gegen momentan empfohlene und bisher noch nicht verwendete Medikamente erlaubt.

Obwohl sich molekulare Resistenzmarker als Frühwarnsystem hilfreich erwiesen haben und verschiedenste Marker und/oder Markerkombinationen für die Überwachung der Resistenz gegen diverse Medikamente schon vorgeschlagen wurden, zeigt die Diskrepanz der Daten, dass auch wichtige andere epidemiologische Faktoren die Resistenzdynamik in einer bestimmten Region bestimmen. Daher wird es zunehmend wichtig, dass bestehende Modelle zur Resistenzdynamik, welche molekulare Daten verwenden, zusätzliche Faktoren einschliessen, um dann in Regionen mit unterschiedlichen Misserfolgsraten der Behandlung und mit unterschiedlichem Medikamentengebrauch getestet zu werden. Abgesehen von der Prävalenz der molekularen Resistenzmarker werden in zukünftigen Modellen auch Indikatoren für andere wichtige Determinanten der Resistenzdynamik, wie zum Beispiel Übertragungsintensität oder Medikamentengebrauch berücksichtigt werden müssen.

# **Chapter 1**

## **Introduction**

## 1. The burden of malaria

Malaria is one of the most important infectious diseases in the world, affecting mainly the tropics and the sub-tropics. At present, about 100 countries or territories are considered malarious, with nearly 50% of them in Sub-Saharan Africa. Globally, approximately 3 billion people corresponding to 40% of the world's population are at risk of infection (Hay *et al.*, 2004). Malaria is a vector-borne parasitic disease caused by intracellular protozoan parasites of the genus *Plasmodium*. Four species, *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, infect humans. The parasites multiply asexually in the human host and go through sexual reproduction in the anopheline mosquito vector (Figure 1). Each type of infection causes debilitating febrile illness, but approximately 90% of clinically manifest infections are caused by *P. falciparum*. *P. vivax* accounts for nearly 10% of the global malaria incidence. The main causes of mortality are severe anaemia and cerebral malaria caused by *P. falciparum*. Recent estimates suggest that between 500 million and 5 billion clinical episodes and up to 3 million deaths occur each year due to malaria, with Sub-Saharan Africa having 90% of this mortality burden. Moreover, the devastating consequences of malaria are a major obstacle to social and economic development in affected regions (Bremner *et al.*, 2004; Mendis *et al.*, 2001; Snow *et al.*, 2005).

In the 1950s and early 1960s, elimination of malaria seemed possible and the World Health Organization (WHO) launched the Global Malaria Eradication campaign with a main focus on indoor residual spraying (IRS) with dichlorodiphenyltrichloroethane (DDT) and mass drug administration (MDA) with chloroquine (CQ) or pyrimethamine (PYR). The eradication strategy was not only abandoned due to logistical, social and political reasons, but mainly because of the occurrence of chemoresistance in both, the vector and the parasite (D'Alessandro & Buttiens, 2001). Thereafter, the world was facing a rapid resurgence of the disease. This has been attributed to several factors, such as the change of agricultural practices creating new vector breeding sites, political crises leading to a weakening of public health systems, and long-term climate changes favouring malaria transmission (Sachs & Malaney, 2002).

In the absence of an effective vaccine, current control efforts of the global partnership program Roll Back Malaria (RBM) clearly focus on reducing malaria morbidity and mortality. Methods include the reduction of transmission by either lowering vector densities using insecticides or biological measures and reducing their contact with humans by the use of insecticide-treated mosquito-nets. A further element is the early detection or forecasting of

malaria epidemics and rapid application of appropriate control measures. But the cornerstone in the control of the disease is the reduction of malaria cases by early diagnosis followed by prompt and effective treatment and prophylaxis of people at greatest risk (i.e., infants and pregnant women) (WHO, 2005a; RBM, 2006).

However, the efficacy of this control strategy is hampered by the emergence and spread of drug resistant malaria which is the major challenge in the control of the disease at present. Therefore, research efforts into the design and development of new antimalarial drugs, which are safe, effective and affordable, have to be sustained. Important measures to prevent or delay the spread of resistance include the protection of currently used and newly introduced drugs by combination therapy (White, 1999) and improvement of access to prompt and effective treatment (Panosian, 2005). Further critical elements to detain resistance include the adoption of adequate methods to assess and monitor drug resistance in order to deploy evidence-based drug policies (Olliaro & Taylor, 2003).

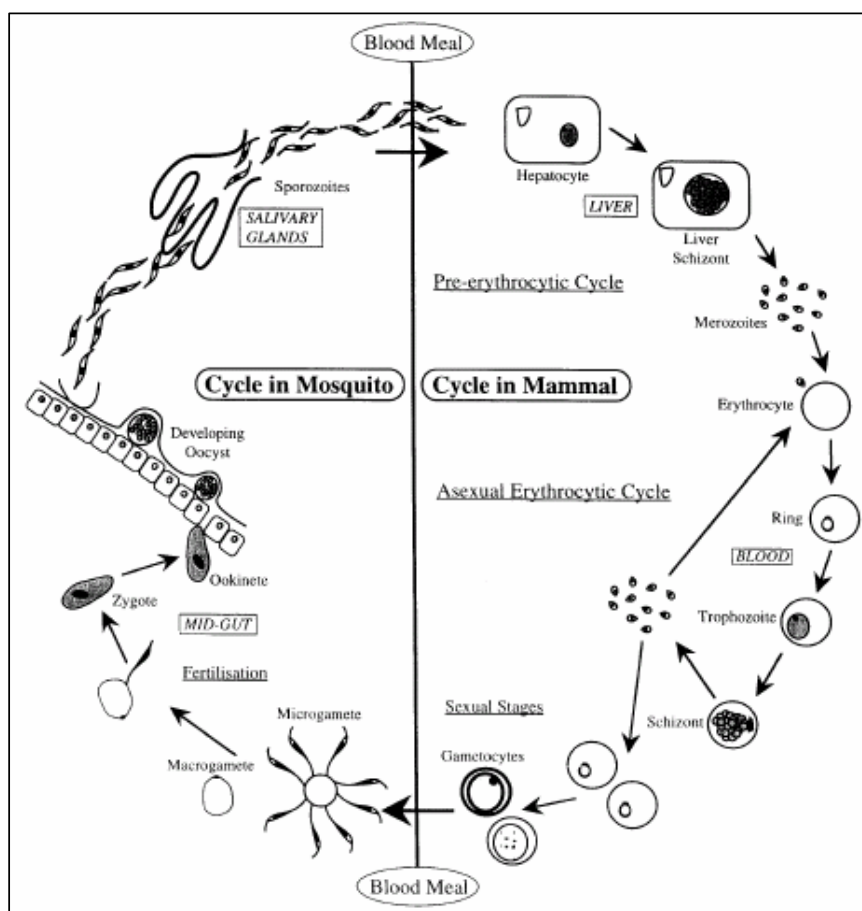


Figure 1: The life cycle of the malaria parasite (Source: Phillips, 2001)

## 2. Antimalarial chemotherapy

The elimination of malaria from most regions in Europe and North America led to a loss of interest in malaria for more than 25 years. Between 1975 and 1999, only 4 of 1393 newly developed drugs were antimalarials (Trouiller *et al.*, 2002). Because of the limited armoury of drugs in endemic countries and a lack of affordable new drugs, malaria control has heavily relied on a restricted number of medicaments mainly belonging to the quinolines and the antifolates. It has been only recently that the artemisinin-based compounds have been introduced widely. Because the useful therapeutic life (UTL) of many of the currently used drugs is severely compromised by drug resistance and newly introduced drugs have to be protected, combination therapy using compounds belonging to different drug classes is strongly recommended (Kremsner & Krishna, 2004; WHO 2001; WHO, 2006). The most common antimalarials used in malaria control programmes, either as mono- or combination therapy and their mode of action are summarised in Table 1.

### 2.1 Quinolines

The 4-aminoquinolines chloroquine (CQ) and amodiaquine (AQ) and the related quinoline methanols quinine (QUIN) and mefloquine (MEF) have been the mainstay of malaria chemotherapy during much of the past 40 years. Halofantrine (HAL), another related phenanthrene methanol, is no longer recommended due to the occurrence of fatal cardiotoxicity, low bioavailability and its high cost (Nosten *et al.*, 1993). The new analogue lumefantrine (LUM) was developed and is now a component of the combination regimen co-artemether (Riamet<sup>®</sup>, Coartem<sup>®</sup>; van Vugt *et al.*, 2000). Primaquine (PRIM) is an 8-aminoquinoline which is highly active against gametocytes of all malaria species in humans and the hypnozoites of the relapsing species *P. vivax* and *P. ovale*. It is recommended as antirelapse treatment and gametocytocidal drug against *P. falciparum* in low or non-transmission areas only (WHO, 2006). Despite extensive studies and the proposition of various mechanisms (reviewed in O'Neill *et al.*, 1998), the mode of action of these drugs is not completely understood. However, the commonly accepted hypothesis is that quinoline-containing drugs accumulate in the digestive vacuole (DV) of the intraerythrocytic parasite and primarily interfere with haemoglobin metabolism. Degradation of haemoglobin by the parasite produces toxic byproducts, the most important being ferriprotoporphyrin IX (FPIX or haem) and free oxygen radicals, which have to be detoxified by a series of parasite-specific processes (Francis *et al.*, 1997). The major mechanisms postulated to be involved in CQ

accumulation are 1) trapping of the weak base CQ in the acidic DV through passive diffusion down a pH gradient, 2) active uptake of CQ by specific transporters, and 3) binding of CQ to the intravacuolar receptor haem (reviewed in Foley & Tilley, 1998). The build-up of CQ-haem complexes subsequently interferes with DV functions eventually leading to parasite death through the following mechanisms. CQ interferes with haem detoxification by the inhibition of its polymerization to  $\beta$ -haematin and sequestration as malaria pigment haemozoin (Bray *et al.*, 1999; Egan *et al.*, 1994; Slater, 1993). The inhibition of peroxidase degradation (Loria *et al.*, 1999) and glutathione-dependent degradation of unpolymerized haem (Ginsburg *et al.*, 1998; Zhang *et al.*, 1999) leads to peroxidative damage of parasite proteins and lipids and membrane disruption. The closely related AQ has been suggested to exert its activity by a similar mechanism (Foley & Tilley, 1998) because efficiency of haem-binding and inhibition of haem-polymerization have been shown to be comparable to CQ (O'Neill *et al.*, 1997; Slater, 1993). Data are conflicting as to whether the mode of action of quinoline/phenanthrene methanols is similar to that of CQ (Foley & Tilley, 1997). However, there are several lines of evidence that the interaction with haem is also central to the activity of QUIN, MEF and HAL (Bray *et al.*, 1999; Mungthin *et al.*, 1998; Slater *et al.*, 1993). Drug action of 8-aminoquinolines seems to be different from that of 4-aminoquinolines (Meshnick & Marr, 1992) and PRIM has been proposed to exert its activity by interfering with mitochondrial function (Beaudoin & Aikawa, 1968; Boulard *et al.*, 1983).

## 2.2 Antifolates

In contrast, the primary targets of another important drug class, the antifolates, have long been established. Antifolates exert their antimalarial action by disruption of the *de novo* biosynthesis of folate, an important co-factor in the amino acid, purine and pyrimidine pathways, which eventually leads to blockage of DNA synthesis and lowered production of glycine and methionine (Krungkrai *et al.*, 1989). There are two important groups of antifolates: 1) the sulpha drugs, such as sulphadoxine (SDX) and dapsone (DAP), which are structural analogues of para-aminobenzoic acid (*p*ABA) and inhibit dihydropteroate synthetase as part of a bifunctional protein with hydroxymethylpterin pyrophosphokinase (PPPK-DHPS), and 2) pyrimethamine (PYR) and proguanil (PG), which is metabolised *in vivo* to the active form cycloguanil (CG), both inhibiting dihydrofolate reductase as part of the bifunctional enzyme with thymidylate synthetase (DHFR-TS) (Yuthavong, 2002). Due to their marked synergistic effect (Chulay *et al.*, 1984), DHFR and DHPS antagonists are mainly

used as combination regimens, the most common being SDX plus PYR (SP, Fansidar™) and PG plus DAP (Lap-Dap™) (Watkins, 1997).

### 2.3 Artemisinins

An important new and entirely different class of compounds originates from the Chinese herb qinghao (*Artemisia annua*) from which the parent compound artemisinin was first isolated in the 1970s. Since then, several analogues, such as dihydroartemisinin, arteether, artemether and artesunate, with better bioavailability have been developed. Artemisinins are endoperoxide-containing sesquiterpene lactones. A number of studies have shown that the endoperoxide-bridge can be cleaved by reductive interaction with iron, yielding free radicals that lead to parasite death, possibly by alkylation of different plasmodial targets (Meshnick *et al.*, 1996; Olliaro *et al.*, 2001). The formation of covalent adducts between artemisinins and haem and several plasmodial and host proteins have been described, but the precise mechanisms involved in antimalarial activity are still to be resolved (Krishna *et al.*, 2004; Meshnick, 2002). More recently, an alternative hypothesis for the mode of action has been proposed, based on structural similarities between artemisinin and thapsigargin, a potent inhibitor of sarcoendoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases (SERCAs) in a variety of organisms (Eckstein-Ludwig *et al.*, 2003). Evidence in favour of this hypothesis included the specific inhibition of the SERCA of *P. falciparum* (PfATPase6) by artemisinins, the interference of thapsigargin with the action of artemisinins, the iron-dependent inhibition of PfATPase6, and the strong positive correlation between inhibition of PfATPase6 and death in cultured parasites. The artemisinins have considerable advantage over other antimalarials because they kill parasites more rapidly and affect a broader range of asexual blood stages (Hien & White, 1993). Unlike 4-aminoquinolines and antifolates, which exert their antiparasitic action on schizonts, artemisinins also impede gametocytes, which limits transmission to new hosts (Price *et al.*, 1996; Targett *et al.*, 2001). Moreover, they inhibit important pathophysiological processes, such as cytoadherence and rosetting, more effectively than other drug classes (Udomsangpetetch *et al.*, 1996).

### 2.4 Other drug classes

Atovaquone (ATQ) is a naphthoquinone derivate and a structural analogue of coenzyme Q (ubiquinone). ATQ acts by specifically binding to the ubiquinone oxidation site in the cytochrome  $bc_1$  complex (CYT  $bc_1$ ) in the electron transport chain and collapsing



mitochondrial membrane potential in the parasite (Srivastava *et al.*, 1999a). Though inappropriate as monotherapy due to rapid selection of resistant parasites, ATQ is clinically successful when used in combination with the synergistically acting partner drug PG (Malarone™) for both, chemoprophylaxis and therapy of *P. falciparum* malaria (Looareesuwan *et al.*, 1999; Hogg *et al.*, 2000; Srivastava *et al.*, 1999b).

A number of antibiotics, such as tetracycline (TET) and doxycycline (DOX), are effective, though slow-acting, antimalarial compounds. They are suggested to inhibit different steps of prokaryote-like protein synthesis in the apicoplast of the parasite (Ralph *et al.*, 2001). However, they are currently used in combination with other drugs or as chemoprophylactic agents in non-immune travellers only (WHO, 2005b).

Table 1: Mode of action of current antimalarial drug classes

Drug class	Members	Target location	Target molecule	Efficacy
Antifolates	PYR, PG SDX, DAP	Cytosol	DHFR, DHPS	Blood-stage schizonticide
Quinolines	CQ, AQ, QUIN, MEF, HAL, LUM PRIM*	Food vacuole	Haem, Others?	Blood-stage schizonticide Gametocytocide* Tissue-stage schizonticide*
Artemisinins	Dihydroartemisinin and derivatives	Food vacuole	<i>Pf</i> ATP6 Others?	Blood-stage schizonticide, Gametocytocide
Naphthoquinones	ATQ	Mitochondrion	Cytochrome <i>bc</i> <sub>1</sub>	Blood-stage schizonticide
Antibiotics	DOX, TET	Apicoplast	Apicoplast ribosome	Blood-stage schizonticide

PYR, pyrimethamine; PG, proguanil; SDX, sulphadoxine; DAP, dapson; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; CQ, chloroquine; AQ, amodiaquine; QUIN, quinine; MEF, mefloquine; HAL, halofantrine; LUM, lumefantrine; PRIM, primaquine; \* PRIM has tissue-schizonticidal and gametocytocidal activity; *Pf*ATPase6, *Plasmodium falciparum* ATPase6; ATQ, atovaquone; DOX, doxycycline; TET, tetracycline

### 3. Drug resistant malaria

#### 3.1 Epidemiology

The parasite's ability to develop resistance affects all currently available drugs except the artemisinin derivatives, although the degree of resistance varies depending on different drugs and regions (Bloland, 2001).

After the introduction in 1943, CQ came into universal use as therapeutic and prophylactic agent against malaria. The success has been based on high clinical efficacy, good safety and tolerability, ease of use and cost-effective production. However, resistance to CQ was first described at the Thai-Cambodian border in the late 1950s (Harinasuta *et al.*, 1965) and in Colombia and Venezuela in the 1960s (Payne, 1987). A further focus emerged in the 1970s in Papua New Guinea (PNG) (Grimmond *et al.*, 1976). In Africa, CQ resistance was first documented in the late 1970s in Kenya (Fogh *et al.*, 1979) and Tanzania (Campbell *et al.*, 1979), and spread first to the central and southern parts before arriving in West Africa in 1983. By 1989, CQ resistance was widespread in Sub-Saharan Africa (Wernsdorfer & Payne, 1991). Today, *P. falciparum* resistance to CQ occurs everywhere except in Central America, the island of Hispaniola and some regions of Southwest Asia (WHO, 2005b). Recent data from population genetic surveys suggest that CQ resistance emerged independently at a limited number of sites: two in South America (Cortese *et al.*, 2002; Wootton *et al.*, 2002), one in PNG (Mehlotra *et al.*, 2001), and one on the Philippines (Chen *et al.*, 2003). These data had shown similarities of parasites from Asian and African origin, but differences from those from South America and PNG, supporting the hypothesis that parasite migration played a critical role in the spread of CQ resistance (Wellems & Plowe, 2001).

Despite the widespread use of CQ, resistance of *P. vivax* has been very limited, apparently having originated in PNG (Rieckmann *et al.*, 1989; Schuurkamp *et al.*, 1992). CQ resistant *P. vivax* malaria may be characterized as endemic to the Indonesian archipelago, sporadic in the rest of Asia, and rare in South America (Baird, 2004).

The spread of CQ resistant malaria has led to increasing use of the combination regimen sulphadoxine-pyrimethamine (SP) as standard first-line regimen in many countries. Antifolate resistance emerged almost instantaneously and independently from several areas where the drug had been introduced on national level. First reported at the Thai-Cambodian border in the late 1960s (Björkman & Phillips-Howard, 1990), high-level SP resistance was rapidly spreading in southeast Asia and the Amazon Basin and moderate frequencies were observed on the Pacific coast of South America, in southern Asia and Oceania (Bloland, 2001). In

Africa, sensitivity started to decrease in the late 1980s, with the highest levels reported from the eastern part of the continent (Wongsrichanalai *et al.*, 2002). Similar to CQ resistance, molecular data suggest that resistance to antifolates has arisen at only a few independent foci and was followed by inter- and intracontinental spread of resistant parasites (Cortese *et al.*, 2002; Nair *et al.*, 2003; Roper *et al.*, 2004).

Reports of clinical resistance to QUIN have been started to accumulate since the mid-1960s, especially from the Thai-Cambodian border. High levels of resistance were described in Thailand in the 1980s, where the introduction of QUIN monotherapy as interim therapy against SP resistant malaria has led to a rapid decrease in sensitivity to the drug (Wernsdorfer, 1994). Therefore, QUIN has been used in combination with other drugs during the following decades and is currently recommended as second-line regimen against uncomplicated malaria and treatment of severe cases only (WHO, 2006).

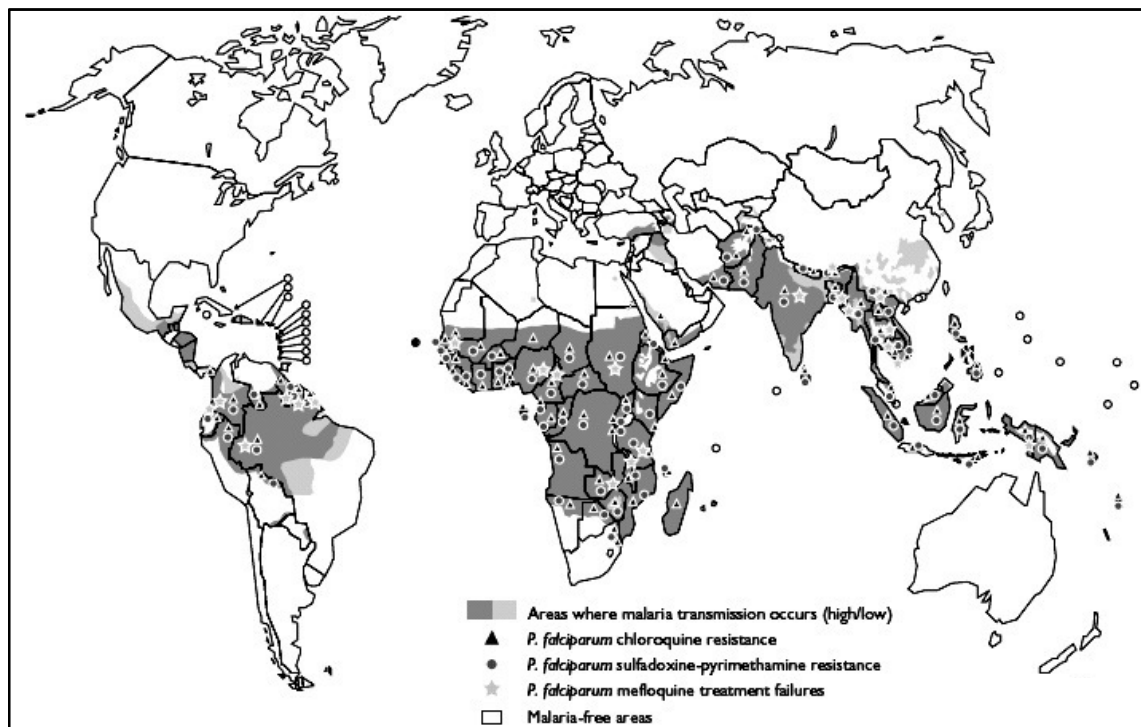


Figure 2: Malaria transmission areas and reported drug resistance in 2004

(Source: WHO, 2005a)

Resistance to MEF was first reported from the Thai-Cambodian border in the late 1980s, five years after it has been introduced (Wongsrichanalai *et al.*, 2001). The high level of MEF resistance in Thailand was most probably due to the heavy use of the chemically related drug QUIN (Brasseur *et al.*, 1992). Though there have been sporadic reports of clinical failure from the Amazon Basin, Bangladesh and India (Wernsdorfer, 1994), and reduced *in vitro* sensitivity of *P. falciparum* strains in Africa has been observed (Jelinek *et al.*, 2001), MEF resistance is rare outside Southeast Asia.

Resistance to ATQ developed in 1996, the same year when the drug was introduced (Looareesuwan *et al.*, 1996) and is currently used as fixed-dose combination with PG (Malarone™) only.

Foci of established multidrug resistant malaria, defined as resistance to more than three operational antimalarial compounds, are found at the border region of Thailand and Cambodia and Myanmar, respectively, and some focal areas in the Amazon Basin (Wongsrichanalai *et al.*, 2002).

### **3.2 Molecular basis of parasite resistance to antimalarial drugs**

Advances in the understanding of the mechanisms of drug action during the last two decades have led to the identification of the putative molecular targets and the genetic basis responsible for parasite resistance to antimalarial drugs. Genetic events conferring resistance include single point mutations in or changes of copy numbers of genes encoding drug targets, such as important enzymes or transporters regulating intraparasitic drug concentrations.

#### **3.2.1 Resistance to quinolines**

Recent molecular analyses strongly argue for multiple genes and epistasis, rather than a single genetic determinant, to be involved in CQ resistance (Anderson *et al.*, 2005; Duraisingh & Refour, 2005). The two main characteristics that distinguish CQ resistant from CQ sensitive parasites are diminished accumulation of CQ in the parasite's digestive vacuole (DV) and reversal of resistance through chemosensitization by verapamil (VP) or other Ca<sup>2+</sup>-channel blockers (Krogstad *et al.*, 1987; Martin *et al.*, 1987). These observations suggested that CQ resistance is most probably associated with altered drug transport processes into the DV and several genes encoding candidate proteins involved in the transport of CQ into or out of the

DV have been proposed: *P. falciparum* multidrug resistance gene 1 (*pfmdr1*), candidate gene 2 (*cg2*), and *P. falciparum* chloroquine resistance transporter (*pfcrt* or *cg10*).

*Pfmdr1*, which is localized on chromosome 5, encodes a P-glycoprotein homolog (Pgh1) and has been localized to the parasite DV (Foote *et al.*, 1989; Wilson *et al.*, 1989; Cowman *et al.*, 1991). Pgh1 has a typical structure shared by members of the ATP binding cassette (ABC) transporter family (Endicott & Ling, 1989). Initial sequence analysis of the full-length *pfmdr1* revealed five polymorphic residues that appear to be dimorphic: N86Y, Y184F, S1034C, N1042D, and D1246Y (Foote *et al.*, 1990a). In the same study, *pfmdr1* mutations were strongly linked to the CQ resistant phenotype, but several subsequent studies failed to confirm the association (Wilson *et al.*, 1993; Basco & Ringwald, 2002). Moreover, analysis of the progeny of a genetic cross between a CQ resistant and a CQ sensitive parasite line found no association between inheritance of the CQ resistant phenotype and the *pfmdr1* locus (Wellems *et al.*, 1990). However, more recent experiments utilizing newly available transfection methods have shown that *pfmdr1* mutations can increase resistance levels to CQ (Reed *et al.*, 2000). In field studies, most attention has been given to the investigation of the *pfmdr1* N86Y allelic variant which is widespread in Africa and Asia. Several studies have demonstrated the selection of the mutant allele following treatment with CQ or AQ (Duraisingh *et al.*, 1997; Sutherland *et al.*, 2002). A positive, though incomplete, association has also been found between *pfmdr1* N86Y and *in vivo* CQ resistance by several authors (Basco *et al.*, 1995; Nagesha *et al.*, 2001; von Seidlein *et al.*, 1997). Nevertheless, numerous other studies have demonstrated contradictory results (Bhattacharya *et al.*, 1997; Basco & Ringwald, 1997; Haruki *et al.*, 1994; Pillai *et al.*, 2001). The allelic variant *pfmdr1* N86Y has not been observed in a large number of South American strains. In contrast, the triple mutation S1034C, N1042D, plus D1246Y is more prevalent on this continent, but was seen in CQ resistant and CQ sensitive parasites (Foote *et al.*, 1990a; Huaman *et al.*, 2004; Pova *et al.*, 1998). Variation in copy number of the *pfmdr1* gene was observed in a number of CQ resistant isolates (Foote *et al.*, 1989; Barnes *et al.*, 1992), but this correlation could not be confirmed in a wide variety of field and laboratory strains (Basco *et al.*, 1995; Wellems *et al.*, 1990).

A series of highly systematic experiments using the progeny of the genetic CQ resistant-CQ sensitive cross led to the discovery of the *cg2* gene family on chromosome 7. Different members were screened for polymorphisms that might correlate with the CQ resistant phenotype in a large array of laboratory-adapted *P. falciparum* strains from around the world. An initially promising candidate, *cg2*, showed a complex pattern of polymorphism that was

tightly, but not perfectly, linked with CQ resistance (Su *et al.*, 1997). Though the weak association between allelic variants of *cg2* and CQ resistance were confirmed by some studies (Basco & Ringwald, 1999), others found no correlation (Sharma *et al.*, 2001). Moreover, transfection experiments, where the *cg2* gene from CQ resistant parasites was replaced with the variant from their CQ sensitive counterparts, showed no effect on the level of CQ resistance in the transformed parasites (Fidock *et al.*, 2000a).

Further analysis of the progeny of the genetic cross of Wellems *et al.* (1990) localized the CQ resistance determinant to a 36kb segment on chromosome 7 (Su *et al.*, 1997; Wellems *et al.*, 1991). Subsequent studies of the segment identified a highly interrupted gene with 13 exons, termed *pfcr*, encoding a putative transporter protein that was localized to the DV membrane of the parasite. Several polymorphisms in *pfcr* showed linkage to the CQ resistant phenotype in a large set of laboratory-adapted *P. falciparum* lines from Africa, South America, and Southeast Asia, but with considerable variations depending on the geographical region (Cooper *et al.*, 2002; Fidock *et al.*, 2000b). Furthermore, an allelic exchange approach replacing the endogenous *pfcr* allele of a CQ sensitive strain with *pfcr* from CQ resistant lines from different origins provided conclusive evidence that mutant haplotypes of the *pfcr* gene product confer CQ resistance with characteristic VP-reversibility and reduced CQ accumulation (Sidhu *et al.*, 2002). The mutation K76T seems to play a major role in determining the CQ resistant phenotype since it was invariably found in all CQ resistant strains so far. The mutation is usually not isolated, but associated with other single nucleotide polymorphisms (SNP) at other codons, C72S, M74I, N75E, H97Q, A220S, Q271E, N326S/D, I356T/L and R371T/I, the role of which is not very well defined. It was suggested that these mutations might play a critical role in maintaining important functional properties of the protein in CQ resistant parasites (Wellems & Plowe, 2001). The importance of the K76T mutation has been further corroborated by several clinical studies which have shown a higher prevalence of the K76T mutation in post-treatment than in pre-treatment samples, which alludes to a strong selection towards the mutant allele under CQ treatment (Djimde *et al.*, 2001a; Schneider *et al.*, 2002). Moreover, the presence of the mutant allele has been shown to be present in all *P. falciparum* isolates which failed treatment with CQ (Basco *et al.*, 2002; Djimde *et al.*, 2001a; Thomas *et al.*, 2002). However, the mutation has also been observed in CQ sensitive isolates (Kyosiimire-Lugemwa *et al.*, 2002) which suggest that either additional mutations in *pfcr* or other genes may be involved in the determination of the CQ resistant phenotype. Interestingly, it has recently been shown that *pfcr* K76T mediated parasite resistance was reversed by concomitant carriage of the *pfcr* mutation S163R, and further

modified by the additional mutation in *pfcr* T152A (Johnson *et al.*, 2004). Currently, there is no evidence that alterations in gene copy number or expression levels of *pfcr* are involved in CQ resistance (Durrand *et al.*, 2004).

Regarding the proposed similar, though not equal, mode of action of the related quinoline drugs MEF, QUIN, HAL and LUM, molecular studies on parasite resistance to these drugs have mainly focused on the two membrane transporter genes *pfcr* and *pfmdr1*. Mutations in *pfcr* have been shown to be associated with resistance to QUIN (Mu *et al.*, 2003). In addition, several *in vitro* studies could demonstrate that point mutations in *pfmdr1* modulate resistance to MEF, QUIN and HAL (Reed *et al.*, 2000; Duraisingh *et al.*, 2000). However, more recent *in vivo* studies conducted in Peru and Gabon did not provide evidence for an association between *pfmdr1* mutations and MEF resistance (Mawili-Mboumba *et al.*, 2002; Pillai *et al.*, 2003). Interestingly, the presence of the *pfmdr1* wild-type allele N86 has been found to be associated with *in vitro* resistance to MEF (Duraisingh *et al.*, 2000; Price *et al.*, 1999) and more recently, with *in vivo* resistance to LUM (Sisowath *et al.*, 2005).

Amplification of the *pfmdr1* gene copy number has been found to be associated with resistance to MEF and HAL in both, laboratory (Cowman *et al.*, 1994, Peel *et al.*, 1994) and field (Price *et al.*, 1999; Wilson *et al.*, 1993) isolates. Though amplification of *pfmdr1* seems not to be a prerequisite for increased MEF resistance (Lim *et al.*, 1996; Chaiyaroj *et al.*, 1999), its important role in predicting *in vitro* and *in vivo* MEF failure has been shown in Thailand (Price *et al.*, 2004). Moreover, recent experiments using *pfmdr1* knockdown clones of the parasite could provide further evidence for this gene modification to be important in mediating resistance to MEF, QUIN, and HAL (Sidhu *et al.*, 2006).

More recent studies have demonstrated that other genes, such as *pfmrp* (multidrug resistance protein) or *pfnhel* (sodium hydrogen exchanger), and as yet not fully characterised loci encoding other transporter molecules, are involved in conferring resistance to quinolines (Ferdig *et al.*, 2004; Klokouzas *et al.*, 2004; Mu *et al.*, 2003). These findings further underscore the current hypothesis that phenotypic resistance to this drug class requires the involvement and interaction of many different genes (Bray *et al.*, 2005; Duraisingh & Cowman, 2005).

Table 2: Genetic changes in *P. falciparum* associated with resistance to quinolines in clinical use as antimalarials

Drug	Gene <sup>a</sup>	Molecular markers <sup>b</sup>
CQ, (AQ)	<i>pfcr</i>	C72S, M74I, N75D/E, <b>K76T</b> , A220S, Q271E
	<i>pfmdr1</i>	<b>N86Y</b> , Y184F, S1034C, N1042D, D1246Y
MEF, QUIN, HAL, LUM	<i>pfmdr1</i>	Copy number > 1; wild-type N86
	<i>pfcr</i>	Mutations affect <i>in vitro</i> resistance to differing degrees in different strains

<sup>a</sup> Genes encoding known targets (it can not be excluded that there are as yet unidentified additional targets); <sup>b</sup> Most commonly observed amino acid changes associated with *in vivo* resistance; **bold**, key mutations. The relative importance of the other mutations (i.e., interference with drug binding or maintenance of protein function) is not yet fully assessed; CQ, chloroquine; AQ, amodiaquine; *pfcr*, *Plasmodium falciparum* chloroquine transporter; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1, MEF, mefloquine; QUIN, quinine; HAL, halofantrine; LUM, lumefantrine

(Source: adapted from Hyde, 2005a)

### 3.2.2 Resistance to antifolates

Though the detailed molecular basis of parasite resistance to antifolates is not yet completely clear, a variety of studies, including genetic analyses, biochemical assays and transfection experiments, have contributed to a better understanding of the molecular events involved in resistance to the individual antifolate compounds.

Resistance to PYR is caused by mutations in the *pfdhfr* gene, which lead to weaker drug binding, but maintain enzyme activity (Chen *et al.*, 1987; Cortese & Plowe, 1998; Sirawaraporn *et al.*, 1997). Several studies have shown the key role of the S108N mutation in conferring the PYR resistant phenotype in *P. falciparum* (Cowman *et al.*, 1988; Peterson *et al.*, 1988). Additional mutations at codons N51I, C59R and I164L progressively enhance



resistance to the drug (Basco & Ringwald, 2000; Wang *et al.*, 1997a). Triple mutants S108N+N51I+C59R are mainly seen in Africa and Southeast Asia, where they are responsible for high level PYR resistance (Hyde, 1990; Sibley *et al.*, 2001). Though relatively uncommon in Southeast Asia and South America (Berens *et al.*, 2003; Biswas *et al.*, 2000; Plowe *et al.*, 1997), and only sporadically reported from single foci in Africa (Alker *et al.*, 2005; Hastings *et al.*, 2002; Staedke *et al.*, 2004), quadruple mutants (plus I164L) represent the severest form of resistance and are responsible for high level resistance to the DHFR inhibitors PYR and CG. The allelic variation A16V coupled with an alternative change at position 108 (S108T) is involved in resistance to CG, with only moderate loss of sensitivity to PYR (Foote *et al.*, 1990b; Peterson *et al.*, 1990). Amino acid changes C50R/I and V140L in *pf dhfr* are rare and were only observed in isolates originating from single foci in South America (Vasconcelos *et al.*, 2000). *Pf dhfr* mutations have been shown to segregate with the drug resistant phenotypes in a genetic cross (Peterson *et al.*, 1988) and final proof for their role in PYR resistance has been obtained by parasite transfection experiments (Wu *et al.*, 1996). Though never been demonstrated *in vivo*, chromosomal rearrangement and gene amplification were demonstrated under drug pressure *in vitro* (Thaithong *et al.*, 2001) and can not yet be ruled out as contributory factors to clinical resistance.

Similarly to *pf dhfr*, resistance to SDX and other sulpha drugs is associated with decreased drug binding and has been linked to mutations in *pf dhps* (Triglia *et al.*, 1999). Amino acid changes at five different sites, S436A/F, A437G, K540E, A581G and A613S/T, have as yet been reported (Brooks *et al.*, 1994; Triglia & Cowman, 1994; Triglia *et al.*, 1997; Wang *et al.*, 1997a). As done for *pf dhfr*, the role of these mutations in conferring resistance to sulpha drugs has been demonstrated in cross-mating and allelic exchange experiments (Triglia *et al.*, 1998; Wang *et al.*, 1997b). Whereas the S108N change in *pf dhfr* seems to be a prerequisite for further accumulation of mutations which results in a progressive increase in PYR resistance, a similar, but less clear-cut, situation is assumed for *pf dhps*, since the A437G mutation, alone or in combination with additional mutations in the gene, predominated in field isolates (Sibley *et al.*, 2001).

An important aspect of antifolate resistance is the rapid selection of resistant parasites due to pharmacologically sub-optimal amounts of drugs persisting in the body after treatment, the reason being that PYR and SDX have long elimination half-lives of 116 h and 81 h, respectively (Diourte *et al.*, 1999; Watkins *et al.*, 1997). Selection pressure exerted by the short-acting antifolates CG and DAP has been shown to be lower (Curtis *et al.*, 1998; Nzila *et al.*, 2000a).

The question how much mutations in *pfdhfr* and *pfdhps* contribute to the level of *in vivo* antifolate resistance has been and still is a matter of debate. Laboratory and field studies dealing with this question are difficult for many reasons. Systematic investigations are hampered by the fact that antifolate drugs are frequently used in combination and act synergistically. Furthermore, numerous different *pfdhfr/pfdhps* haplotypes are observed in field samples (Plowe *et al.*, 1997). However, there is ample evidence for a positive correlation between the number of mutations in both genes and the level of prior SP usage. In the Middle East for instance, where little SP has been used, all isolates had wild type *pfdhfr* and *pfdhps*, whereas most isolates from Southeast Asia were highly mutated in both genes (Wang *et al.*, 1997a). Also a number of sites in Africa, where SP has been widely used within the last decade, reported high prevalence rates of triple-*pfdhfr* plus double-*pfdhps* genotypes, such as in Northern Tanzania where rates up to 60% were measured in community surveys (Pearce *et al.*, 2003).

The overall tendency is to consider that the triple-*pfdhfr* mutation could be a useful genetic marker for *in vivo* resistance to SP and that point mutations in *pfdhps* play a secondary role in determining treatment failure (Basco *et al.*, 1998; Mockenhaupt *et al.*, 2005; Mugittu *et al.*, 2004). However, there are several other authors who claim mutations in *pfdhps* to be equally or even more important in predicting treatment response to SP (Berens *et al.*, 2003; Dorsey *et al.*, 2004). Reports are conflicting because host factors confound the association between molecular resistance markers and *in vivo* drug response. In addition, the investigation of a relationship is further complicated by the fact that many, but not all, *Plasmodium* strains have the ability to use exogenous folate from the host. This salvage pathway (i.e., exogenous folate utilization via a pathway that obviates the need for DHPS), which is believed to provide only a minority of folate production in the parasite, the majority being produced by *de novo* biosynthesis, can be blocked by PYR (Wang *et al.*, 1997b; Wang *et al.*, 1999). This might not only be a possible explanation for the observed synergy of drug action between PYR and SDX, it could also be a putative explanation for the asymmetric selection of mutations in *pfdhfr* and *pfdhps*, which has been demonstrated in many *in vitro* and field studies (Mberu *et al.*, 2000; Nzila *et al.*, 2000b; Plowe *et al.*, 1997). Selection for mutations in *pfdhfr* occurs first and mutations in *pfdhps* are only selected if parasites carry at least a double mutation in *pfdhfr*. It therefore seems that mutations in *pfdhps* become important once resistance in *pfdhfr* has reached a degree where therapeutic levels of PYR are not sufficient anymore to kill the parasite by the inhibition of DHFR alone (Sims *et al.*, 1999). Though the exact genetic basis for this ‘folate effect’ is not fully elucidated at this time, current molecular hypotheses assume

the locus for the differences in folate utilisation to be closely linked to *pfdhfr* (Wang *et al.*, 1997b; Wang *et al.*, 2004). However, data about the prevalence of this capacity in natural parasite populations and its contribution to a SP resistant *in vivo* phenotype are still scarce (Dzinjalama *et al.*, 2005). More recently, the conversion of SDX by DHPS to sulpha-pterin adducts, which have inhibitory effects further downstream the folate biosynthesis pathway, has been shown (Mberu *et al.*, 2002). The effect was independent of mutations in *pfdhfr* or *pfdhps* and led to the hypothesis that sulpha drugs could inhibit the parasite by mechanisms other than the blockage of DHPS and therefore, resistance could be mediated by other genes (Patel *et al.*, 2004). Moreover, the assumption that these drug adducts have detrimental effect on folate synthesis led to the speculation that sulpha drug resistant parasites may be selected on the basis of lower production of these toxic adducts rather than reduced competition for binding to DHPS (Hyde, 2005b).

Table 3: Genetic changes in *P. falciparum* associated with resistance to antifolates in clinical use as antimalarials

Drug	Gene <sup>a</sup>	Molecular markers <sup>b</sup>
PYR	<i>pfdhfr</i>	C50R, N51I, C59R, <b>S108N</b> , I164L
PG (CG)	<i>pfdhfr</i>	A16V, N51I, C59R, <b>S108T/N</b> , I164L
SDX, DAP	<i>pfdhps</i>	S436A/F, <b>A437G</b> , K540E, A581G, A613S/T

<sup>a</sup> Genes encoding known targets (it can not be excluded that there are as yet unidentified additional targets); **bold**, key mutations. The relative importance of the other mutations (i.e., interference with drug binding or maintenance of enzyme function) is not yet fully assessed; <sup>b</sup> Most commonly observed amino acid changes associated with *in vivo* resistance; PYR, pyrimethamine; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; PG, proguanil; CG, cycloguanil; SDX, sulphadoxine; DAP, dapsone; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase

(Source: adapted from Hyde, 2005a)

### 3.2.3 Resistance to other drug classes

Atovaquone (ATQ) has been shown to inhibit the cytochrome *bc*<sub>1</sub> (CYT *bc*<sub>1</sub>) complex of the electron transport chain of malaria parasites. Mutations in *P. falciparum* CYT *bc*<sub>1</sub> were associated with ATQ resistance *in vitro* (Korsinczky *et al.*, 2000) and were reported in a small number of *in vivo* failures with Malarone™ (Fivelman *et al.*, 2002; Wichmann *et al.*, 2004). Interestingly, mutations in CYT *bc*<sub>1</sub> were found to be associated with a loss of fitness in *P. falciparum*, which could suggest that the prevalence of resistant parasites may decrease after drug usage is discontinued (Peters *et al.*, 2002).

Up to date, clinical resistance to artemisinin and its derivatives, when used in combination with other drug classes, has not yet been observed. However, there are several lines of evidence that drug resistance to this drug class emerges. These include 1) a clinical study that demonstrated decreased *in vitro* sensitivity to artemisinin in *P. falciparum* isolates from patients who failed treatment with artesunate monotherapy (Menard *et al.*, 2005), 2) experiments that showed increased *in vitro* resistance to artemether for isolates from French Guiana (Jambou *et al.*, 2005), and 3) a report of the development of genetically stable and transmissible resistance to artemisinin and its derivatives in *P. chabaudi chabaudi* (Afonso *et al.*, 2006). Most current models suggest a multiple-target model for the mode of action of artemisinins (Golenser *et al.*, 2006), and several findings from molecular studies investigating parasite resistance to this drug class are in support of this hypothesis. Drug assays using mutant *pfcr*t lines showed that these CQ resistant strains were also slightly less susceptible to AQ, but more susceptible to QUIN, MEF and artemisinin (Duraisingh *et al.*, 2000; Sidhu *et al.*, 2002). This implicates *pfcr*t to govern parasite susceptibility to a variety of compounds including the newly introduced drug class of artemisinins. Likewise, increased copy number of *pfmdr1* has been found to be associated with artemisinin resistance in isolates from patients who failed treatment with combination therapy of MEF plus artesunate (Price *et al.*, 2004). More recently, molecular studies have found mutations at codons 263 and 769 in *pfATPase6*, a putative target for artemisinin derivatives, to be associated with decreased drug susceptibility *in vitro* (Jambou *et al.*, 2005; Uhlemann *et al.*, 2005).

These findings further emphasise the need to use artemisinins in combination with other companion drugs to which high-grade resistance has not yet been developed, in order to prolong the useful therapeutic life (UTL) of this highly effective drug class (Duffy & Sibley, 2005; WHO, 2006). At the same time, continued research efforts are needed, not only to

consolidate recent molecular findings, but also further elucidate the genetic basis of drug action of and parasite resistance to artemisinin.

Table 4: Genetic changes in *P. falciparum* associated with resistance to other drug classes in clinical use as antimalarials

Drug	Gene <sup>a</sup>	Molecular markers <sup>b</sup>
ATQ <sup>c</sup>	CYT <i>bc</i> <sub>1</sub>	Y286S/N
Artemisinin and derivates <sup>d</sup>	<i>pfmdr1</i>  <i>pfATPase6</i> <sup>e</sup>	Status of <i>pfmdr1</i> modulates level of <i>in vitro</i> sensitivity  L263, S769N, (E431K, A623E)
DOX, TET	<i>Pf</i> mitochondrion	Not yet characterised

<sup>a</sup> Genes encoding known targets (it can not be excluded that there are as yet unidentified additional targets); <sup>b</sup> Most commonly observed amino acid changes associated with *in vivo* resistance; <sup>c</sup> only in clinical use in combination with proguanil (Malarone™); <sup>d</sup> clinical resistance to artemisinin-based combination regimens not yet observed; <sup>e</sup> shown by *in vitro* experiments only; ATQ, atovaquone; CYT *bc*<sub>1</sub>, cytochrome *bc*<sub>1</sub> complex; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1; *pfATPase6*, *Plasmodium falciparum* ATPase 6; DOX, doxycycline; TET, tetracycline

(Source: adapted from Hyde, 2005a)

### 3.3 Assessment of resistance

#### 3.3.1 Classical tools

Two methods have been widely used to assess the level of drug resistant malaria:

- I. *In vivo* drug efficacy studies which are based on WHO standardised Day 7, 14, or 28 monitoring of patients after treatment for uncomplicated malaria using parasitology alone (S/RI/RII/RIII levels of resistance), or parasitology and symptoms (adequate clinical response, early or late treatment failure) as outcome(s) (Table 5), and
- II. *In vitro* assays to measure intrinsic sensitivity of *P. falciparum* parasites to antimalarial drugs.

At present, *in vivo* therapeutic tests still remain the gold standard for monitoring antimalarial drug efficacy and guiding drug policy. These *in vivo* test systems were developed and introduced shortly after the first reports of CQ resistance in 1965. Thereafter, protocols were simplified, revised and standardised for the assessment of antimalarial drug efficacy in infants and young children in areas with high malaria transmission (WHO, 1996). Though the methodology has recently been revised and includes now several modification and adaptations for low to moderate transmission areas as well as a common classification for all transmission areas (WHO 2002; WHO, 2003), it is still a subject of debate with no consensus on several issues, such as length of follow-up, etc. (White, 2002). Apart from several advantages of *in vivo* studies, such as the generation of easily interpretable results and a minimal requirement for equipment and supplies, they are afflicted with several drawbacks. These include the interference of host factors (e.g. immunity or drug uptake and metabolism) with treatment outcome, strongly reduced compliance because of long follow-up periods, the assessment of resistance to one drug regimen only, and poor ability to compare different studies because local adaptations and modifications of the standard protocol are usually made.

Table 5: Definitions of *in vivo* therapeutic efficacy and parasitological resistance

<b>Parasitological resistance outcomes (WHO, 1996)<sup>a</sup></b>	
<b>Sensitive</b>	Asexual parasite count reduces to 25% of the pre-treatment level within 48 hours after initiation of treatment and complete clearance on Day 7, without subsequent recrudescence up to Day 28
<b>RI</b>	Asexual parasitaemia reduces to <25% of pre-treatment level within 48 hours after initiation of treatment, but reappears between Day 7 and Day 28
<b>RII</b>	Marked reduction in asexual parasitaemia (decrease >25% but <75%) within 48 hours after initiation of treatment, without complete clearance on Day 7
<b>RIII</b>	No or minimal reduction in asexual parasitaemia, (decrease <25%) or an increase in parasitaemia within 48 hours after initiation of treatment
<b>Therapeutic efficacy outcomes (WHO, 2003)<sup>b</sup></b>	
<b>ETF</b>	<ul style="list-style-type: none"> <li>• Danger signs or severe malaria on Day 1, 2, or 3, or</li> <li>• Parasitaemia on Day 2 higher than on Day 0, or</li> <li>• Parasitaemia on Day 3 <math>\geq 25\%</math> of Day 0 count, or</li> <li>• Parasitaemia on Day 3 with axillary temperature <math>\geq 37.5^{\circ}\text{C}</math></li> </ul>
<b>LCF</b>	<ul style="list-style-type: none"> <li>• Danger signs or severe malaria after Day 3, or</li> <li>• Parasitaemia with axillary temperature <math>\geq 37.5^{\circ}\text{C}</math> (or history of fever) from Day 4 to Day 28</li> <li>• Without meeting any of the criteria for ETF</li> </ul>
<b>LPF</b>	<ul style="list-style-type: none"> <li>• Parasitaemia from Day 7 to Day 28 without axillary temperature <math>\geq 37.5^{\circ}\text{C}</math> (or history of fever)</li> <li>• Without meeting any of the criteria for ETF or LCF</li> </ul>
<b>ACPR</b>	<ul style="list-style-type: none"> <li>• Absence of parasitaemia on Day 28,</li> <li>• Without meeting any of the criteria for ETF, LCF or LPF</li> </ul>

<sup>a</sup> Outcomes for extended test protocol (i.e., Day 14 or Day 28 follow-up); R, resistance; <sup>b</sup> Protocol based on Day 14 follow-up for high transmission areas and Day 28 follow-up for low to moderate transmission areas; ETF, Early treatment failure; LCF, Late clinical failure; LPF, Late parasitological failure; ACPR, Adequate clinical and parasitological response

Several *in vitro* assay systems are available and differ with regard to end-point measurement. These include the WHO mark III test (microscopic examination of blood films for assessment of schizont maturation), radioisotopic tests (incorporation of radio-labelled nucleotide precursors), ELISA (Enzyme-linked immunosorbent assay) tests based on antibodies against *Plasmodium* lactate dehydrogenase (production of enzyme) or histidine-rich protein 2 (secretion of soluble antigen) (reviewed in Noedl *et al.*, 2003). *In vitro* assays have several advantages, such as the ability to assess simultaneously several drugs (including experimental compounds) and host confounding factors are avoided. However, the main problems encountered with *in vitro* sensitivity tests are that they require expensive equipment and supplies, there is partial lack of standardised protocols, threshold values for resistance are not determined for all drugs, and the correlation with therapeutic efficacy tests is not yet fully established. Therefore, they are not readily amenable to large scale epidemiological mapping, especially in low-resource countries, and are recommended to be used as adjunct to *in vivo* efficacy studies (WHO, 2005b).

### **3.3.2 New tool: molecular monitoring of parasite resistance**

Advances in the understanding of the mechanism of drug action allowed the identification of the putative molecular targets responsible for resistance. At present, the assessment of molecular markers is still considered as complementary tool for monitoring antimalarial resistance (WHO, 2003; WHO, 2005b). Though the advantages of the molecular tests are similar to those of *in vitro* assays (i.e., detection of true parasite resistance without interfering host factors, ability to perform multiple analyses with a single patient sample), they are limited with regard to many aspects. Most of the work has been focused on CQ and SP, both available and cheap drugs widely used in malaria endemic areas worldwide. Therefore, molecular markers are only available for a limited number of drugs. Furthermore, the role of molecular markers in predicting *in vivo* therapeutic outcome has been controversial and the correlation has not been fully established. Presently, a number of methods exist for SNP analysis in antimalarial drug resistance genes, but each has its disadvantages. Many are based on PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis of selected loci or on sequence specific amplification or hybridisation (Ranford-Cartwright *et al.*, 2002; Sangster *et al.*, 2002). These techniques are limited in the number of samples and SNPs which can be analysed simultaneously. They are usually quite costly and often limited by available restriction sites. Other available techniques, such as MALDI-TOF



based systems (Marks *et al.*, 2004), pyrosequencing (Nair *et al.*, 2002), molecular beacons (Durand *et al.*, 2000), real-time PCR (Alker *et al.*, 2004; de Monbrison *et al.*, 2003), or clamped-probe PCR (Senescau *et al.*, 2005), are also prohibitively expensive and complex. Taken together, there is a lack of standardised protocols for sample collection and DNA extraction as well as standard operating procedures (SOP) for further downstream molecular analyses. In addition, most of these technologies require a high infrastructure laboratory and equipment and supplies which are expensive. Hence, they are not feasible in many field laboratories in countries with limited resources. In order to provide a monitoring system based on the analysis of various SNPs in a timely manner on a large scale (i.e., numerous populations and sites), new tools have to be developed and validated.

## **4. Rationale, aims and objectives**

### **4.1 Rational of the current study**

One of the greatest challenges for health authorities of malaria endemic countries is to decide on when and how drug policy should be changed (i.e., at which level of parasite resistance or treatment failure should a malaria control program change the first-line drug). A number of criteria, such as the level of parasite resistance (RIII resistance 5-30%) (Bloland, *et al.*, 1993; Sudre *et al.*, 1992) or *in vivo* treatment failure (total failure rate  $\geq 25\%$  and clinical failure rate  $\geq 15\%$ ) (WHO 2005b; WHO, 2006) have been suggested. But even if these criteria are agreed on, several questions remain:

- I. What is the best way to assess these outcomes?
- II. How do these outcomes differ from one country to the other (i.e., between different epidemiological settings)?
- III. Which alternative drug(s) should be used?
- IV. How should the development of parasite resistance to newly introduced and/or withdrawn drug(s) be monitored?

The difficulties in determining parameters for drug policy change are mainly attributable to the limitations of the currently applied tools. The routine methods for the assessment of resistance (i.e., *in vivo* drug efficacy studies and *in vitro* sensitivity tests) are demanding in terms of personnel, equipment, costs and time. Moreover, the lack of standardised protocols and procedures applicable to different epidemiological settings makes comparability between studies difficult. In recent years, the growing body of knowledge about the molecular

mechanisms involved in parasite resistance has led to the advocacy for molecular monitoring of parasite resistance as a supplementary tool to *in vivo* drug efficacy studies (Plowe, 2003; Quaye & Sibley, 2002; WHO, 2003). The role of several point mutations at different loci in conferring resistance *in vivo* has mainly been inferred from studies showing predictive association of particular mutations with treatment failure and from overrepresentation of mutations in recrudescence infections after treatment. However, on an individual level, an association of specific molecular markers with *in vitro* resistance does not allow prediction of resistance *in vivo* or of the therapeutic response. A number of other parameters, such as the use of drug combinations, the level of prior immunity which is closely linked to transmission intensity, compliance to treatment, etc., play a role in clearing symptoms and parasites (White, 2004). Hence, direct proof of a causal relationship between single or a combination of markers and clinical failure has remained elusive and most models now postulate a multigenic basis of resistance (Anderson *et al.*, 2005; Mu *et al.*, 2003). As yet, such clinical-based studies were conducted in a relatively small number of sentinel sites and at infrequent time intervals because of the high cost and resources required. In addition, these studies usually investigated symptomatic patients in a restricted age group (i.e., children  $\leq 5$  years of age) and were thus assessing a biased sub-sample of the whole parasite population. In most instances, the large parasite reservoir circulating in the untreated asymptomatic population, which might play an important role in the development and spread of parasite resistance, was not investigated.

The analysis of point mutations on population level and the establishment of correlations between the molecular drug resistance profile in parasites and *in vivo* parasitological and clinical outcome could give a more comprehensive appraisal of the status and longitudinal dynamics of resistance. Several population-based molecular surveys have already been conducted, but only two studies in Mali (Djimé *et al.*, 2001b) and Uganda (Talisuna *et al.*, 2002; Talisuna *et al.*, 2003) have investigated the correlation with the level of clinical failure in health facilities of the same region. Moreover, these studies have only assessed one locus, which considerably limits the conclusions that can be drawn for further drug policy implementation.

Studies on an epidemiological scale are mainly limited by the currently applied methods for the analysis of molecular resistance markers. Although the procedures to determine these drug-related parasite genotypes are relatively simple and already in use in several laboratories in sub-Saharan Africa, Asia, and South and Central America, their capacity to analyse multiple SNPs in several genes simultaneously is limited. Moreover, in the few studies that dealt with this issue, haplotype frequencies have been estimated from individuals with single

infections, discarding the ones with multiple infections. Since a vast majority of parasitaemic individuals harbour multiple infections, especially in areas of high transmission, this approach is of only limited applicability. For all these reasons, an accurate and complete picture of the genetic resistance pattern in an area is difficult to get.

In the current project, we evaluate a new approach that should overcome these problems. We make use of a high throughput system to analyse all known molecular markers of resistance in samples collected in community-based cross-sectional surveys. By using different combinations of SNPs in several marker genes we should be able to assess the relationship between the genetic drug resistance background in the parasite population and treatment failure rates more accurately. A series of rapid assessments using cross-sectional surveys of a sub-sample of the general population could be performed in several sites and the frequencies of point mutations (and combinations of mutations) in multiple marker genes in the community could then be used to predict the clinical response at health facilities. If reproducible results can be obtained in different geographical areas with different levels of endemicity, rapid age-balanced cross-sectional surveys in the community could eventually allow overcoming resource-consuming *in vivo* studies to assess the level of *Plasmodium* resistance to various drug regimens. These surveys could also be conducted in remote areas that are not well served by health facilities and have been left aside in the past. An informed and rationale decision could thus be made by health authorities on the best time to change drug policy and on the best drug to choose. Moreover the monitoring of the development of resistance to the newly introduced drug(s) as well as of the reversal of resistance to previous drug(s) abandoned could be assessed. Finally, quick surveys for the longitudinal assessment of frequencies of markers would allow to capture the dynamics of resistance and to construct predictive models for different areas with different levels of transmission.

## 4.2 Aim and specific objectives

This study aimed to bring directly together data from community-based molecular surveys and data derived from health centre-based studies. Since the routine tools for the assessment of antimalarial resistance (i.e., *in vivo* and *in vitro* studies and current methods for the analysis of molecular resistance markers in parasites) are too cumbersome and difficult to use on a large scale, we assessed the relevance of a new appraisal approach: Community-based cross-sectional surveys versus clinical studies (population versus patient), and the usefulness of a new molecular technology (DNA microarray versus PCR-RFLP or sequencing) for the

identification of relevant SNPs in different parasite genes. The frequencies of SNPs in given resistance marker genes as well as genotype patterns were analyzed in clinical samples to investigate their role in predicting *in vivo* treatment response. Furthermore, corresponding community drug resistance profiles were correlated with the incidence risk of clinical treatment failure in order to investigate the relevance and usefulness of such a novel approach in the management of drug use.

The specific objectives of the present PhD project were as follows:

1. To assess the treatment efficacy of sulphadoxine-pyrimethamine (SP) plus amodiaquine (AQ) or chloroquine (CQ) against uncomplicated *P. falciparum* and *P. vivax* malaria in Papua New Guinea (Chapter 2)
2. To develop and validate a DNA microarray-based technology for the assessment of molecular markers of drug resistant malaria (Chapter 3)
3. To assess the genetic drug resistance profile in clinical samples and to determine useful molecular markers in *P. falciparum* for predicting treatment outcome with combination therapy of AQ or CQ plus SP (Chapter 4)
4. To test the hypothesis that the parasite population circulating in the community has the same genetic profile of resistance markers as the population circulating in malaria patients attending health facilities (Chapter 5)
5. To estimate the frequency and patterns of molecular markers in community samples and to compare them with the incidence risk of clinical failure in the health centres serving these communities (Chapter 6)
6. To assess the dynamics of molecular markers in the parasite population under standard treatment regimen over a period of two to three years (Chapter 6)
7. To assess and investigate the role of molecular markers in *P. vivax* for predicting treatment outcome with combination therapy of AQ or CQ plus SP (Chapter 7)

## 5. Study design and methodology

### 5.1 Study area

Studies were conducted in Papua New Guinea (PNG) which is a patchwork of different geographical and ecological zones and inhabited by a population of approximately 5.7 million people characterized by exceptional cultural and linguistic diversity. PNG features complex variations in vector and malaria epidemiology. All four *Plasmodium* species that infect humans are found in both, lowland and highland areas, with *P. falciparum* and *P. vivax* being the predominant species. Malaria intensity ranges from unstable low-level endemicity where outbreaks are common to high transmission comparable with most endemic regions in Sub-Saharan Africa. Malaria is the commonest cause of outpatient presentation and accounts for 27% of all attendances at health facilities (Müller *et al.*, 2003). Studies were conducted between October 2002 and March 2005 at three different sites in PNG. These sites included:

- I) The Sigimaru health centre (HC) in the Karimui area (Simbu Province), a rural region in the highland fringe area of PNG located at an altitude of 700 to 1200 m,
- II) The Kunjingini HC in the South Wosera area (East Sepik Province), a remote region located in the floodplain of the Sepik river in the North-eastern part of the country, and
- III) The Mugil HC at the North Coast of the country (Madang Province), a rainforest region located near the provincial capital town of Madang (Figure 3).

Though malaria transmission is perennial with limited variations between wet (October to April) and dry (May to September) season at all three sites, areas cover different levels of transmission intensity and drug use patterns. Transmission intensity decreases significantly with increasing altitude (Müller *et al.*, 2003) and is higher in the lowland regions of the Wosera and the North Coast than in the Karimui area. There is little socioeconomic stratification between and within sites, with most of the inhabitants being subsistence farmers, but there are differences with regard to health care provision and drug use patterns (Benet *et al.*, 2004; Genton *et al.*, 1995; Hii *et al.*, 2001; Mehlotra *et al.*, 2002; Müller *et al.*, 2004).

Scientific approval and ethical clearance for the studies were obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG. Informed consent was first requested from all the communities involved and prior to recruitment, individual consent was obtained from each study participant and parents or legal guardians.

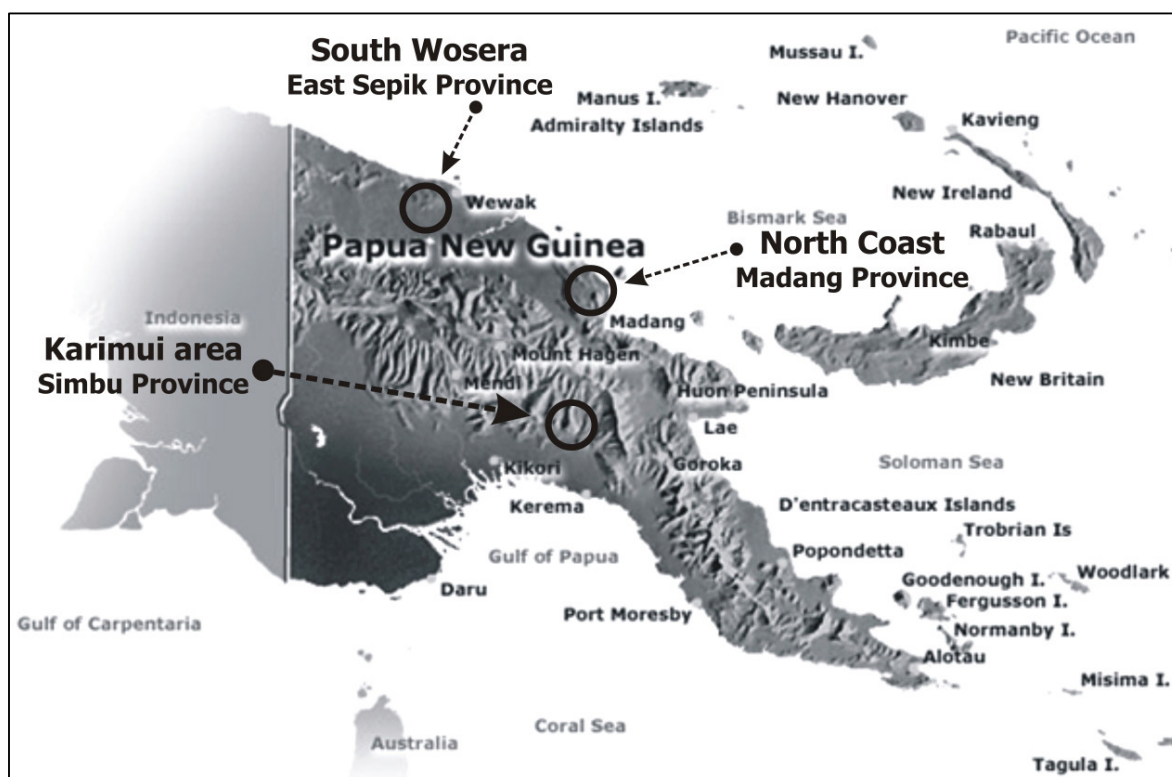


Figure 3: Map of Papua New Guinea presenting the location of study sites

## 5.2 Methods

The project used data collected at two different levels:

- I) Repeated *in vivo* drug efficacy studies were conducted at the three health centres over the period of three consecutive years to estimate the incidence risk of treatment failure with the current first-line regimen of AQ or CQ plus SP against uncomplicated malaria. We used the newly revised WHO standard protocol for low to moderate transmission areas (WHO, 2003) and applied genotyping methods for the distinction of recrudescences and new infections and corrected failure rates accordingly (Cattamanchi *et al.*, 2003; Slater *et al.*, 2005). The method is described in detail in Chapter 2 and study forms are found in Appendix II.
- II) Repeated community-based cross-sectional surveys were conducted in the catchment areas of the corresponding health centres in order to determine the

frequencies and patterns of drug resistance markers in a random sample of the parasite population. Apart from clinical assessment, collected information included demographic characteristics, history of sickness (onset, type and duration of symptoms), health facility attendance, purchase or consumption of drugs outside health facilities, and antimalarial treatment courses received in the preceding year. Methodology is described in detail in Chapters 5 and 6. Study forms and characteristics of the study populations are found in Appendix II and IV, respectively.

Molecular analyses were done for:

- I) The assessment of the number of infecting clones per sample. The multiplicity of infection (MOI) was determined by genotyping the highly polymorphic *msp2* (merozoite surface protein 2) locus of *P. falciparum* (Felger and Beck, 2002).
- II) The assessment of the molecular drug resistance profile in clinical and community samples. For this purpose, we used a newly developed DNA microarray-based technology. The method is described in detail in Chapter 3 and standard operating procedures (SOP) are found in Appendix III.

## 6. References

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## Chapter 2

### **Low efficacy of amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against falciparum and vivax malaria in Papua New Guinea**

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## ABSTRACT

Due to increasing resistance to 4-aminoquinolines in Papua New Guinea (PNG), combination therapy of amodiaquine (AQ) or chloroquine (CQ) plus sulphadoxine-pyrimethamine (SP) was introduced as first-line treatment against uncomplicated malaria in 2000. It was the aim of this study to assess the *in vivo* efficacy of the combination therapy against *P. falciparum* and *P. vivax* malaria.

Studies were conducted between 2003 and 2005 in the Simbu, East Sepik and Madang Provinces in PNG according to the revised protocol of the World Health Organization (WHO) for the assessment of antimalarial drug efficacy. Children aged 6 months--7 years presenting with a clinically overt and parasitologically confirmed *P. falciparum* or *P. vivax* malaria were monitored up to Day 28 and classified according to clinical and parasitological outcome as ACPR (Adequate clinical and parasitological response), ETF (Early treatment failure), LCF (Late clinical failure), and LPF (Late parasitological failure).

Treatment failure rates for *P. falciparum* malaria up to Day 28 ranged between 16% and 30%, depending on the region and the year of assessment. The corresponding PCR-corrected values after differentiation of true recrudescences from new infections by parasite genotyping were 12% and 28%, respectively. Overall treatment failure rate in *P. vivax* malaria was 12%.

Our results suggest that the current first-line treatment in PNG is not sufficiently effective. According to the new WHO guidelines for the treatment of malaria, a rate of parasitological resistance above 10% in the two dominant malaria species in the country justifies a change in treatment policy.

## INTRODUCTION

Malaria is a serious health problem in Papua New Guinea (PNG) and access to safe and effective treatment still remains the mainstay in the control of the disease. The 4-aminoquinoline drugs amodiaquine (AQ) and chloroquine (CQ) have been first-line treatment against uncomplicated malaria until the late 1990s. However, resistance of *Plasmodium falciparum* to CQ was first documented in 1976<sup>1,2</sup> and numerous studies done since then in different provinces at different times showed the problem to be widespread. Within two decades, resistance to the 4-aminoquinolines AQ and CQ increased gradually with a slow shift from RI to RII and RIII types.<sup>3--9</sup> The first documented evidence for *P. vivax* resistance in PNG was reported in 1989<sup>10,11</sup> and showed a similar increasing trend as for *P. falciparum*.<sup>12</sup>

Although pyrimethamine in combination with CQ has been used in mass drug administration campaigns in the 1960s,<sup>13</sup> the combination of sulphadoxine-pyrimethamine (SP) was not previously part of the standard treatment against uncomplicated malaria and was used only in combination with quinine to treat severe or treatment failure malaria in the country. Despite the low use of SP, resistance of *P. falciparum* to this drug combination was first described in PNG in 1980.<sup>14</sup> Thereafter, *P. falciparum*-resistance to SP as well as reduced efficacy of SP against *P. vivax* has been reported in the Madang province.<sup>15--18</sup>

In view of the low efficacy of the 4-aminoquinolines used as first-line regimen against malaria, PNG health authorities were prompted to revise antimalarial treatment policy in 1997. Combination therapy for uncomplicated malaria has been advocated for some years to improve clinical effectiveness and to delay the development and spread of resistance to the individual drugs.<sup>19,20</sup> Though evidence for the success of the combination regimen of AQ or CQ plus SP was scarce at that time<sup>21</sup> and there was evidence for *in vivo* as well *in vitro* resistance against either of the drugs in the country, the decision to investigate the possible change to this combination regimen was made. Based on efficacy trials conducted between

1998 and 1999, that showed that the combinations were efficacious with treatment failure rates below 5%,<sup>22</sup> the PNG Department of Health chose these combination regimens to replace the monotherapy with AQ or CQ as the standard first-line treatment against uncomplicated malaria in the year 2000.

Since the introduction of the new drug policy, little data has been collected on the efficacy of the new standard treatment. Two studies conducted at health facilities in Maprik and Madang in 2001 recorded treatment failure rates up to Day 14 of 3% and 8%, respectively (JC Reeder, personal communication). These data showed clinical resistance to the combination regimen only one year after introduction and this has been further substantiated by more recent molecular studies showing a high prevalence of mutations in CQ resistance associated marker genes *Pfcr1* (*P. falciparum* chloroquine resistance transporter gene) and *Pfmdr1* (*P. falciparum* multidrug-resistance gene 1), and also a low prevalence of mutations in the gene encoding *Pfdhfr* (*P. falciparum* dihydrofolate reductase) known to confer resistance to SP.<sup>23,24</sup>

Therefore, the principal aim of this study was to assess the status of the clinical efficacy of the current first-line regimen of AQ or CQ plus SP against *P. falciparum* and *P. vivax* malaria in PNG after its official implementation in the year 2000. Within the framework of a project for the clinical and molecular monitoring of drug resistant malaria in PNG, we conducted *in vivo* drug efficacy studies in three different areas in PNG between 2003 and 2005.

## MATERIALS AND METHODS

**Patients and study sites.** The studies were conducted at the Sigimaru health centre (HC) in the Karimui area (Simbu Province), the Kunjingini HC in the South Wosera area (East Sepik Province), and the Mugil HC in the North Coast area of the Madang Province. In Karimui, the studies were run between October and April in three consecutive years (2002, 2003, and 2004). In the Wosera, the study period was between December and June in two following years (2003 and 2004). The study at the North Coast was conducted between April 2004 and February 2005. Though all the three areas are rural places and endemic for malaria, they differ with regard to malaria epidemiology, level of health care provision and history of drug use.<sup>25--30</sup>

Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and informed consent was obtained from parents or legal guardians prior to recruitment of each patient.

**Assessment of drug efficacy.** Drug efficacy studies were conducted according to the standardised WHO protocol for low to moderate transmission areas.<sup>31</sup> Briefly: children between 6 months and 7 years of age were enrolled if they were presenting at the health centre with a microscopically confirmed *Plasmodium* infection (*P. falciparum* density > 1000 asexual parasites per microlitre of blood, *P. vivax* density > 250 asexual parasites per microlitre of blood) and clinically overt malaria (axillary temperature  $\geq 37.5^{\circ}\text{C}$  or history of fever during the last 24 hours for *P. falciparum*, or fever during the last 48 hours for *P. vivax*). *P. falciparum* cases were enrolled regardless of whether they had a concomitant infection with any other *Plasmodium* species, whereas a mixed infection with another species was an exclusion criterion for the *P. vivax* group. However, in mixed *P. falciparum* plus *P. vivax* infections, drug action was evaluated against both parasites species.

Further inclusion criteria were the absence of danger signs for severe or complicated malaria<sup>32</sup> and no signs of any other disease, malnutrition or anaemia. Standard AQ or CQ plus SP first line-treatment (10 mg amodiaquine or chloroquine per kg on Day 0, 1 and 2, and 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 0) was administered under supervision over the first three days. Follow-up visits were scheduled on Day 1, 2, 3, 7, 14, and 28. On every visit, patients were clinically examined and a Giemsa-stained blood slide was taken for the microscopic assessment of parasitaemia.

Patients were advised to come to the health centre on any other day if symptoms occurred. Whenever a child was diagnosed as treatment failure, standard second-line treatment (5 mg artesunate per kg on Day 1 followed by 2.5 mg artesunate per kg on Day 2 to 7, and a single dose of 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 3) was given.

A patient was withdrawn from the study when any of the following occurred during the follow-up period: development of a concurrent infection requiring treatment, consumption of other antimalarial drugs, or loss of follow-up due to refusal of consent or failure to trace a patient on a follow-up visit.

**Molecular analyses.** Blood samples were taken on Day 0 (pre-treatment sample) and on Day 14 and 28 or any day of treatment failure for molecular genotyping purposes. Differentiation between recrudescence and new infection with *P. falciparum* was achieved by comparing PCR-RFLP generated genotype patterns of the merozoite surface protein 2 (*msp2*) in pairs of samples obtained at enrolment and at the day of reappearance of parasitaemia, as described elsewhere.<sup>33,34</sup>

**Data analysis.** Data were double entered in EpiData software (version 3.02, Odense, Denmark) and analysis was performed using STATA software (version 8.2; Stata Corp., College Station, Texas). Patients in the *P. falciparum* group were classified according to their clinical and parasitological responses as follows: Early Treatment Failure (ETF, parasitaemia on Day 2 higher than on Day 0 or parasitaemia on Day 3 with axillary temperature  $\geq 37.5^{\circ}\text{C}$ ), Late Clinical Failure (LCF, parasitaemia with axillary temperature  $\geq 37.5^{\circ}\text{C}$  or history of fever from Day 4 to Day 28), Late Parasitological Failure (LPF, parasitaemia from Day 7 to Day 28 without axillary temperature  $\geq 37.5^{\circ}\text{C}$  or history of fever) or Adequate Clinical and Parasitological Response (ACPR, absence of parasitaemia on Day 28 without meeting any of the previously described criteria for early or late treatment failure). For the *P. vivax* group, a patient was classified as treatment failure (TF) when 1) clinical deterioration due to *P. vivax* malaria in the presence of parasitaemia, or 2) parasitaemia between Day 3 and Day 28 with axillary temperature  $\geq 37.5^{\circ}\text{C}$ , or 3) parasitaemia between Day 7 and Day 28, irrespective of clinical conditions, was observed.<sup>35</sup>

Logistical regression analysis was used for the investigation of possible risk factors for treatment failure and frequencies were compared by using chi-squared tests or Fisher's exact tests as applicable.

## RESULTS

***In vivo* drug efficacy against *P. falciparum*.** From a total of 687 children eligible for the *P. falciparum* group, 38 (5.5%) were excluded from the analysis population. There were no exclusions due to the occurrence of a concomitant infectious disease during the study period. All losses were due to withdrawal of consent of parents or guardians during the follow-up period (3.6%) or migration and/or absence of families after Day 3 (1.9%). Therefore, clinical and parasitological monitoring up to Day 28 was accomplished for a study group of 649 (94.5%) children. The baseline characteristics of the children at day of enrolment were similar for the three study sites and the corresponding years, except for mean parasite density at Day 0 (Table 1). Mean parasite densities were higher in the Wosera ( $t = 2.80$ ,  $p \leq 0.01$ ) and the North Coast area ( $t = 3.10$ ,  $p \leq 0.01$ ) than in Karimui.

At enrolment, 555 children (85.5%) presented with a monoinfection with *P. falciparum* and 94 (14.5%) with a mixed infection. Among the mixed infections, 86 (91.5%) were simultaneously infected with *P. vivax*, 7 (7.4%) with *P. malariae*, and 1 (1.1%) with both of the latter species.

Standard treatment was given under supervision over the first three days and the decision on whether children received SP in combination with AQ or CQ was dependent on weight (AQ for <14 kg). CQ plus SP was given to 128 (19.7%) children (median age of 6 years), whereas 521 (80.3%) children were treated with AQ plus SP (median age of 4 years).

A summary of the classification of the treatment outcomes for *P. falciparum* at the three sites in the different years is shown in table 2. In the Karimui area, treatment failure rates up to Day 28 decreased over the three-year period from 30% to 25%, and 18%, respectively. This trend remained even after PCR-correction (28%, 18%, and 16%, respectively), which identified 11%, 26%, and 9% of recurrences to be new infections ( $\chi^2_{(2)} = 4.81$ ,  $p = 0.09$ ). The overall decreasing trend in treatment failure rates over the study period



was especially so because of a decrease in clinical failures. In the South Wosera area, overall failure rate tended to increase from 2003 to 2004 ( $\chi^2_{(2)} = 1.19, p = 0.28$ ), from 19% in 2003 to 28% in 2004, and after genotyping correction from 16% to 22%, respectively, with 24% of recurrences in 2003 and 33% in 2004 being new infections. Treatment failure rate up to Day 28 in 2004 was 16.4% in the North Coast area of Madang, 11.5% after PCR-correction with 29% of recurrent parasites being new infections.

From the total of 120 (18.5%) treatment failures in the *P. falciparum* group, 97 (80.8%) had a monoinfection with *P. falciparum*, 20 (16.7%) a mixed infection with *P. vivax*, and 3 (2.5%) a mixed infection with *P. malariae* at Day 0. In our study, none of known risk factors (i.e., age, fever or parasite density at day of enrolment) or the combination regimen (SP plus AQ or CQ, respectively) were associated with an increased risk of treatment failure. A mixed infection with *P. vivax* and/or *P. malariae* at Day 0 showed a slightly increased risk of *P. falciparum* treatment failure (OR=1.53), but this effect did not reach statistical significance ( $p = 0.11$ ). Recurrent parasitaemia with other species was seen in 36 (5.5%) of all cases, in 34 (5.2%) with *P. vivax*, and in 2 (0.3%) with *P. malariae*. Ten of the 86 patients (11.6%) with a mixed infection with *P. vivax* at Day 0 had recurrence with *P. vivax*, therefore representing *P. vivax* failure cases (Table 3). From all patients with a *P. falciparum* monoinfection at Day 0, 24 (4.3%) had recurrence with *P. vivax*, and one (0.2%) with *P. malariae*. From the two patients with recurrent *P. malariae*, one had a monoinfection with *P. falciparum*, the second a mixed infection with *P. falciparum* and *P. vivax* at Day 0. The patient with a mixed infection with all three species at enrolment had no recrudescence parasitaemia during the follow-up period.

***In vivo* drug efficacy against *P. vivax*.** To maximize the sample size for analysis, data from the *P. vivax* groups enrolled at all study sites between 2004 and 2005 were pooled. From

a total of 106 children with a *P. vivax* monoinfection at admission day, two were lost due to withdrawal of consent. At baseline, the analysis population had a mean age of 3 years (95% CI = 2.7--3.4), a mean axillary temperature of 37.7°C (95% CI = 37.4--38.0), a mean haemoglobin level of 10.1 g/dl (95% CI = 9.7--10.5), and a mean parasite density of 4182 asexual parasites per microlitre whole blood (range = 40--50640). CQ plus SP was given to 5 (4.8%), AQ plus SP to 98 (94.2%) children, and one (1.0%) was treated with primaquine plus SP. *P. vivax* treatment failure, defined as recurrent parasitaemia after Day 3 irrespective of clinical symptoms, was seen in 13 (12.5%) of all children (Table 3). There was a significant difference of failure rates between sites ( $\chi^2_{(2)} = 13.95$ ,  $p = 0.001$ ): 10/34 (29.4%) *P. vivax* infections in the North Coast area of Madang and 3/46 (6.5%) in the Karimui area failed treatment, whereas all 27 infections were successfully cleared in the Wosera area. Recurrent parasitaemia with *P. falciparum* was observed in two (1.9%) patients who had both successfully cleared their *P. vivax* infection.

## DISCUSSION

In Papua New Guinea, standard first-line therapy with AQ or CQ against uncomplicated malaria was replaced with the combination regimen of AQ or CQ plus SP in the year 2000. The current studies conducted between 2003 and 2005 were the first ones to assess the therapeutic efficacy of the newly introduced combination regimen against *P. falciparum* and *P. vivax* malaria using the revised WHO standard protocol. In our studies conducted in three different areas over the period of three consecutive years, we observed PCR-corrected treatment failure rates up to 28% for *P. falciparum* and 12% for *P. vivax* malaria.

There is strong advocacy for artemisinin-based combination therapy (ACT).<sup>36</sup> However, for economic reasons, many countries have decided on combination regimens including more affordable options, such as AQ or CQ plus SP. PNG replaced 4-aminoquinoline monotherapy with AQ or CQ plus SP in 2000, a decision which was based on efficacy levels above 95% assessed by trials using the 14 day follow-up protocol. When we restricted the analysis in our studies to the Day 14 outcomes based on clinical and parasitological criteria only, we measured treatment failure rates between 2% and 18%. As expected, failure rates up to Day 28 were higher, with PCR-corrected values between 12% and 28%, depending on the area and the year. In concordance with previous data, our results show that *in vivo* studies with a follow-up period of 14 days are not sensitive enough to assess the therapeutic efficacy of the current first-line regimen in moderately to highly endemic areas.<sup>37,38</sup> Assessment up to Day 14 clearly underestimates the true failure rate because in the majority of patients, recurrent parasitaemia appeared after Day 14. Furthermore, late recurrences (i.e., appearing after Day 14) have to be expected for regimens including drugs with long elimination half-lives, such as SP.<sup>39</sup>

Our results show a two to threefold decrease in efficacy of AQ or CQ plus SP only three years after successful implementation of the new first-line regimen. Though clinical failure rates were still low (< 10% at all three sites), resistance levels exceeded 12% in all three sites. It is commonly accepted that parasitological response should be used as an additional indicator for the *in vivo* efficacy of drugs. Parasitological failure rates are likely to translate into clinical failure rates, either within a short term in the infected individual depending on the immunological status, or within a long term on population level as parasite resistance increases.<sup>35,40</sup> Moreover, according to the new WHO guidelines, which recommend that a policy change should be seriously considered when efficacy of a combination regimen up to Day 28 is below 90%,<sup>40</sup> these high levels of *in vivo* resistance are worrisome.

Surprisingly, the dynamics of drug efficacy over time in the Karimui and the Wosera area showed contrasting trends. Whereas treatment failure rates showed a decreasing trend over three years in Karimui, they showed an increasing trend over two years in the Wosera. The fact that the decreasing trend in Karimui was mainly attributable to a drop in clinical failures up to Day 14 without a change of failures between Day 14 and 28, might suggest the quality of the drug batches (i.e., mainly SP) used in Karimui in 2003 to be considerably lower than those in the following years. Otherwise, the question remains whether our observations are the product of intrinsic regional variations or reflect real trends in the dynamics of resistance in these areas. We are aware that the sample sizes and the time intervals between the studies might not have been sufficient to detect real trends. Supplementation of the *in vivo* results with additional molecular data, the most important being the level of resistance in the circulating parasite population (Marfurt J. and others, unpublished data), and ongoing monitoring activities will give further indications about the level and dynamics of drug resistant *P. falciparum* malaria in PNG.

In view of the history of drug use in PNG, the observation of increasing failure rates with AQ or CQ plus SP is not surprising. In the face of increasing CQ resistance, many countries in Africa and Asia had adopted SP as first-line antimalarial between the 1960s and the 1980s.<sup>41</sup> Thereafter, several countries facing increasing levels of SP resistance had introduced the cheap and safe combination of AQ or CQ plus SP as interim option for antimalarial therapy. Whereas SP combined with AQ had shown a reduction in clinical as well as total failure rates up to Day 28, the combination with CQ has not been associated with much benefit over monotherapy with SP.<sup>42,43</sup> Considering the high levels of resistance to AQ and CQ in PNG and a known history of antifolate use in the country before the introduction of the combination regimen, the rapid appearance of resistance had to be expected, because an added benefit of combination therapy is heavily dependent on pre-existing efficacy of the partner drugs.<sup>44</sup> And since resistance levels to AQ and CQ have been known to be high in PNG, it was very unlikely that these drugs would have had sufficient capacity to significantly curb the development of resistance to SP and therefore prolong its useful therapeutic life.

CQ resistance of *P. vivax*, the second dominant species in PNG, was first described in 1989<sup>11</sup> and treatment failure rates up to 20% for CQ and 8% for AQ were reported from Maprik in the late 1990s.<sup>6</sup> Reduced sensitivity of *P. vivax* malaria to SP has been observed in Madang.<sup>17</sup> When we evaluated the therapeutic efficacy of AQ or CQ plus SP in 190 patients with a *P. vivax* infection, we measured a total treatment failure rate up to Day 28 of 12%. Relapse is an important aspect of *P. vivax* malaria and refers to clinical malaria caused by reappearing parasites which originate from the dormant liver stages called hypnozoites. Therefore, circulating asexual stages after blood schizonticidal therapy might either originate from asexual parasites that survived therapy, from activated hypnozoites which lead to a relapse, or from a new infection. Unlike with *P. falciparum* infections, where true recrudescences can be distinguished from new infections by the use of genotyping methods,<sup>45</sup> current molecular methods used for the genetic analysis of *P. vivax*<sup>46-49</sup> do not allow the

unambiguous classification of recurrent parasitaemia, in particular the distinction between a relapse originating from an antecedent infection and a newly acquired infection during the follow-up period, which is critical in the analysis of the therapeutic response. However, recent work on the establishment of standard protocols, similar to those developed for *P. falciparum* including multiple polymorphic genes, look promising and might be included in future drug efficacy trials.<sup>50</sup> Though patients in our study were exposed to the risk of a new infection during the follow-up period and parasite genotyping methods were not applied, we have good reason to assume that our data represent true *P. vivax* resistance to treatment. Former studies demonstrating that no *P. vivax* relapses occurred until Day 36 after full compliance to treatment with the long half-life drug CQ, most probably due to minimal effective concentrations of the drug preventing a first relapse to become patent in the blood, led to the proposition that parasitaemia recurring within 28 days after initiation of CQ therapy reflects resistance to the drug.<sup>51</sup> This concept might be even more relevant with a combination regimen containing CQ and a second long half-life drug, such as SP.<sup>52</sup>

It has long been thought that SP is less active against *P. vivax* malaria, an assumption which was mainly based on clinical studies failing to demonstrate SP efficacy against this species.<sup>53</sup> Accordingly, SP has never been recommended for *P. vivax* malaria. Nevertheless, increasing levels of resistance of *P. vivax* to CQ led to the introduction of SP in many countries in South East Asia, Central and South America and other parts of Oceania, where both species are endemic, and resistance had developed rapidly in many areas within only a few years after its initial deployment as monotherapy.<sup>54,55</sup> It has been shown recently that the mechanisms of *P. vivax* resistance to antifolates are similar to those of *P. falciparum*. Several studies have reported an association between single nucleotide polymorphisms in *P. vivax* dihydrofolate reductase (*Pvdhfr*) and reduced sensitivity to SP.<sup>56–59</sup> Moreover, *in vivo* studies conducted in areas with previous history of SP use against *P. falciparum* have shown that SP resistance of *P. falciparum* was paralleled by the development of resistance of *P. vivax*.<sup>52,60</sup>

These findings further argue for a similar mechanism of antifolate resistance in both species, one that is driven by exertion of selective drug pressure and progresses rapidly. It does therefore not come as a surprise that despite the addition of SP, *P. vivax* failure rates rose from 8% with AQ monotherapy<sup>6</sup> to 12% with AQ or CQ plus SP combination. High level of *P. vivax* resistance is however still restricted to the Madang area, where *P. vivax* resistance even exceeds the one seen in *P. falciparum*.

Occurrence of *P. falciparum* parasitaemia was seen in two (2%) of all *P. vivax* patients. Cryptic coinfections with *P. falciparum* after treatment against *P. vivax* malaria have been described in other areas where both species are endemic.<sup>61,62</sup> Since appearance of *P. falciparum* in both cases was seen after two weeks of treatment, it may reflect acquisition of a new infection. However, a more plausible explanation is that a concomitant *P. falciparum* infection was not recognized at day of admission. This could have occurred because the *P. falciparum* infection was in its hepatic stage, or due to difficulties in differentiating the erythrocytic stages of the two species by microscopy. In contrast, occurrence of *P. vivax* in patients with a *P. falciparum* monoinfection at admission day was seen in 24 (4.3%) of all patients. *P. vivax* parasites appeared between Day 7 and 28, suggesting that patients had a concomitant infection with both species at presentation, either as patent infection which was not detected by microscopical diagnosis, or as relapse from intra-hepatic infection shortly after initiation of treatment against *P. falciparum*. In both cases, recurrent parasitaemia would represent resistance since circulating drug levels should have eliminated drug sensitive parasites.

In conclusion, the high parasitological failure rates of *P. falciparum* and *P. vivax* to the combination therapy with AQ or CQ plus SP only after a short time of successful implementation suggest that the current first-line regimen in PNG is not sufficiently effective and that a policy change needs to be considered. Whilst further monitoring assessing

molecular markers for parasite resistance to CQ and SP, and also other drugs, such as the artemisinin derivatives, is ongoing in PNG, clinical trials to test the safety and efficacy of alternative replacement regimens are urgently needed so that a policy change can be rapidly initiated. *P. falciparum* and *P. vivax* malaria are both endemic in PNG and in most health facilities, antimalarial therapy is given based on presumptive clinical diagnosis. Therefore, apart from safety, tolerability, practicability and cost, efficacy to both of the prevailing species is an important aspect to consider in the evaluation of any future combination regimen.



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TABLE 1: Baseline characteristics of children with a *P. falciparum* infection at admission day

Study site	Karimui area (Simbu Province)			South Wosera area (East Sepik Province)		North Coast area (Madang Province)
	2003 (n = 97)	2004 (n = 93)	2005 (n = 128)	2003 (n = 112)	2004 (n = 115)	2004 (n = 104)
Characteristics						
Weight (mean (95% CI), kg)	14.3 (13.5-15.1)	15.7 (14.4-16.9)	17.1 (15.8-18.3)	14.6 (14.0-15.3)	16.9 (14.3-19.6)	14.7 (11.7-17.7)
Age (mean (95% CI), yrs)	4.0 (3.7-4.4)	4.2 (3.9-4.6)	4.4 (4.2-4.7)	4.5 (4.2-4.8)	4.7 (4.5-5.1)	3.5 (3.2-3.7)
Sex: females/n (%)	F: 43/97 (44.3)	F: 51/93 (54.8)	F: 60/128 (46.9)	F: 59/112 (52.7)	F: 57/115 (49.6)	F: 43/104 (41.4)
Temperature (mean (95% CI), °C)	38.7 (38.5-38.9)	38.7 (38.5-38.8)	38.5 (38.4-38.7)	38.7 (38.4-39.0)	38.0 (37.8-38.3)	38.0 (37.8-38.3)
Hb (mean (95% CI), g/dl)	9.0 (8.6-9.5)	9.5 (9.1-10.0)	9.6 (9.2-9.9)	9.0 (8.7-9.3)	8.8 (8.5-9.1)	9.3 (8.8-9.7)
Parasite density (geometric mean (range), per µl)	21937 (1120-329400)	23786 (1040-187440)	19364 (1000-238880)	40526 (280-774400)	37244 (1000-512000)	38706 (1160-261160)

CI, Confidence interval; Hb, haemoglobin

TABLE 2: Treatment outcomes for amodiaquine (n = 521) or chloroquine (n = 128) plus sulphadoxine-pyrimethamine against *P. falciparum* malaria

Study site		Karimui area (Simbu Province)			South Wosera area (East Sepik Province)			North Coast area (Madang Province)
Year	2003	2004	2005		2003	2004		2004
Class (no (%))	(n = 97)	(n = 93)	(n = 128)	$p(\chi^2_{(2)})$	(n = 112)	(n = 115)	$p(\chi^2)$	(n = 104)
Follow-up to Day 14								
ACPR	80 (82.47)	80 (86.02)	126 (98.44)		96 (85.71)	94 (81.74)		97 (93.27)
TF	17 (17.53)	13 (13.98)	2 (1.56)		16 (14.29)	21 (18.26)		7 (6.73)
Follow-up to Day 14, PCR corrected								
ACPR	80 (82.47)	83 (89.25)	126 (98.44)		98 (87.50)	96 (83.48)		97 (93.27)
TF	17 (17.53)	13 (10.75)	2 (1.56)	<0.001	14 (12.50)	19 (16.52)	0.39	7 (6.73)
Follow-up to Day 28								
ACPR	67 (69.07)	70 (75.27)	105 (82.03)		90 (80.36)	83 (72.17)		87 (83.65)
ETF	2 (2.06)	0 (0)	0 (0)		5 (4.46)	11 (9.57)		0 (0)
LCF	7 (7.22)	0 (0)	0 (0)		1 (0.89)	0 (0)		4 (3.85)
LPF	21 (21.65)	23 (24.73)	23 (17.97)		16 (14.29)	21 (18.26)		13 (12.50)
TF	30 (30.39)	23 (24.73)	23 (17.97)		22 (19.64)	32 (27.83)		17 (16.35)
Follow-up to Day 28, PCR corrected								
ACPR	70 (72.16)	76 (81.27)	107 (83.59)		94 (83.93)	90 (78.26)		92 (88.46)
ETF	2 (2.06)	0 (0)	0 (0)		5 (4.46)	11 (9.57)		0 (0)
LCF	7 (7.22)	0 (0)	0 (0)		1 (0.89)	0 (0)		4 (3.85)
LPF	18 (18.56)	17 (18.28)	21 (16.41)		12 (10.71)	14 (12.17)		8 (7.69)
TF	27 (27.84)	17 (18.28)	21 (16.41)	0.09	18 (16.07)	25 (21.74)	0.28	12 (11.53)

ACPR, Adequate clinical and parasitological response; ETF, Early treatment failure; LCF, Late clinical failure; LPF, Late parasitological failure;

TF, Treatment failure

TABLE 3: Treatment outcomes for amodiaquine (n = 174) or chloroquine (n = 16) plus sulphadoxine-pyrimethamine against *P. vivax* malaria

<b>Class (no (%))</b>	<b>Study site</b>	<b>Karimui area</b> (Simbu Province)	<b>South Wosera area</b> (East Sepik Province)	<b>North Coast area</b> (Madang Province)	$p(\chi^2_{(2)})$	<b>Total</b>
<b><i>P. vivax</i> monoinfections</b>		(n = 43)	(n = 27)	(n = 34)		(n = 104)
<b>ACPR</b>		40 ( <b>93.0</b> )	27 ( <b>100</b> )	24 ( <b>70.6</b> )		91 ( <b>87.5</b> )
<b>TF</b>		3 ( <b>7.0</b> )	0 ( <b>0</b> )	10 ( <b>29.4</b> )	0.001	13 ( <b>12.5</b> )
<b>Mixed <i>P. vivax</i> plus <i>P. falciparum</i> infections</b>		(n = 36)	(n = 32)	(n = 18)		(n = 86)
<b>ACPR</b>		33 ( <b>91.7</b> )	29 ( <b>90.6</b> )	14 ( <b>77.8</b> )		76 ( <b>88.4</b> )
<b>TF</b>		3 ( <b>8.3</b> )	3 ( <b>9.4</b> )	4 ( <b>22.2</b> )	0.29	10 ( <b>11.6</b> )

ACPR, Adequate clinical and parasitological response; TF, Treatment failure



## Chapter 3

### **A rapid and field applicable microarray-based method for monitoring of all single nucleotide polymorphisms associated with parasite resistance to antimalarial drugs**

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## ABSTRACT

*Plasmodium falciparum* resistance to drugs is a public health concern and monitoring of drug efficacy is part of national health systems. Drug resistance is mostly conferred by SNPs. To monitor the spread of these mutations techniques are required facilitating analyses of multiple SNPs. We report a rapid and affordable microarray technique for application in epidemiological studies on malaria drug resistance.

All known resistance-associated SNPs in *pfdhfr*, *pfdhps*, *pfcr1*, *pfmdr1*, and *pfATPase6* genes were analysed by a single tube mini-sequencing reaction and subsequent microarray hybridisation. After evaluation using sequenced parasite material, naturally infected samples from Papua New Guinea were analysed. There was overall consistency of over 90% and analysis by microarray can be done with <10 parasites.

This fast and cost-effective monitoring system facilitates longitudinal monitoring as early warning system and can be applied to detect re-emergence of drug susceptible parasites after withdrawal of a drug.



## INTRODUCTION

Parasite resistance to antimalarial drugs has become a major public health concern for endemic areas and a threat to malaria control programs (1). Therefore, monitoring of antimalarial drug efficacy has become an integral part of national health systems. Efficacy of first-line antimalarial drugs is currently monitored primarily by *in vivo* methods. Such investigations pose major problems in terms of recruitment, costs, and adherence to follow-up visits. In highly endemic areas, efficacy studies are confounded by new infections during the follow-up period (2). In addition, such a monitoring system does not allow the determination of efficacy of drugs which have been discontinued as a result of decreased efficacy, and whose efficacy has been reported to re-emerge once drug selection pressure had ceased (3, 4). *In vivo* studies may also fail to describe the true drug resistance situation in a country because they are usually based on a small and often highly biased sample from the population (5).

Single nucleotide polymorphisms (SNPs) have frequently been associated with susceptibility to disease (6), with differences in drug metabolism (7), and with reduced sensitivity to drugs in microorganisms (8). Drug resistance of the malaria parasite *Plasmodium falciparum* is nearly always conferred by several SNPs. Resistance to sulfadoxine-pyrimethamine (SP) is conferred by point mutations at codons A16V, N51I, C59R, S108N/T, and I164L in the *dhfr* (dihydrofolate reductase) gene. Resistance is augmented by point mutations in the *dhps* (dihydropteroate synthase) gene (S436A, A437G, K540E, A581G, and A613T/S) (9). Multiple SNPs in the transporter genes *mdr1* (multidrug resistance gene 1) and *crt* (chloroquine resistance transporter) have been implicated in resistance to 4-aminoquinolines (10, 11). Recently, mutations in the plasmodial *ATPase6* gene have been associated with decreased susceptibility to artemisinins (12).

Because molecular monitoring of parasite drug resistance has a potential to become a complementary tool for long-term surveillance and for developing predictive models on malaria drug resistance (13, 14), a technique is required that facilitates parallel

analysis of multiple SNPs. It must be affordable and applicable for studies at epidemiological scale.

Presently, a number of methods exist for SNP analysis, but each has its limitations. Many are based on PCR-RFLP analysis of selected loci or on sequence specific amplification or hybridisation (15). These techniques have limitations in the availability of diagnostic restriction sites or the vulnerability to false positive signals. In addition, costs are high for these techniques and throughput is low. Other methods such as MALDI-TOF (16), pyrosequencing (17), real-time PCR (18, 19), clamped-probe PCR (20), or molecular beacons (21), are also prohibitively expensive for epidemiological studies. Consequently, many previous studies have analysed only a few SNPs deemed as primary predictors of resistance. Little attention has been paid to mutations that are not directly associated with resistance, but are considered to have modulating or compensatory effects.

In order to overcome these limitations, we have developed a parallel SNP analysis system for monitoring parasite drug resistance in malaria. Our aim was to develop a user-friendly technology that can rapidly and accurately detect multiple SNPs on a large scale at low cost. We validated our approach with markers of parasite resistance to antimalarial drugs where important criteria are simplicity and robustness to allow transfer to countries with limited resources.

We present here a microarray-based system to determine all known SNPs in drug resistance associated *P. falciparum* genes. In relation to previously used techniques, costs are significantly lower and large numbers of samples can be analysed in a reasonably short time. We have already shown that this technique can be transferred and run in laboratories with minimal infrastructure (22, 23). This technology is also flexible and adaptable for many other applications requiring SNP analyses.

## MATERIAL AND METHODS

Our method is based on parallel PCR amplification of the target sequences followed by primer extension mediated mini-sequencing using fluorochrome-labelled ddNTPs. Subsequent base calling occurs on a microarray upon sequence specific hybridisation. The flow chart in figure 1 depicts schematically the principle of the parallel SNP analysis system.

### Analysed material

To establish and evaluate the technique both cultured material from strains 3D7 and K1, and samples collected during community surveys in Papua New Guinea have been used.

### Blood samples and DNA preparation

Blood samples were collected in EDTA Microtainer™ tubes (BD Biosciences, Allschwil, Switzerland), plasma was separated by centrifugation and red blood cell pellets were stored frozen until used. DNA from cultures and field samples was extracted using QIAamp® DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

### DNA amplification

We analysed the following 36 polymorphisms in 5 genes at 32 SNP sites: *pfmdr1* codons N86Y, Y184F, S1034C, N1042D and D1246Y, *pfcr1* codons C72S, K76T, H97Q, T152A, S163R, A220S, Q271E, N326D/S, I356L/T and R371I, *pfdhfr* condons A16V, N51I, C59R, S108N/T and I164L, *pfdhps* codons S436A, A437G, K540E, A581G, A613T/S, I640F, and H645P, and *pfATPase6* codons S538R, Q574P, A623E, N683K, and S769N. Oligonucleotides for amplification, extension, and arraying are shown in supplementary table 1. To cover all SNP sites, we performed 10 PCR reactions with amplification primers listed in supplementary table 1. The amplification reaction contained 1 x PCR buffer with MgCl<sub>2</sub> in a final concentration of 3 mM, 0.2 mM dNTPs, and 0.2 µM of each primer. Reactions were carried out in 50

µl containing 2.5 µl DNA and 2.5 U *Taq* polymerase (Firepol<sup>®</sup>, Solis BioDyne, Tartu, Estonia). Cycling conditions were: 96°C for 3 min followed by 20 cycles of 96°C for 30 sec, 52°C for 90 sec, and 72°C for 90 sec.

As our aim was to identify SNPs also in asymptomatic samples from community-based surveys we performed nested PCR for highest sensitivity. Nested PCR reactions were carried out in 100 µl with 5 µl primary PCR products and 5 U *Taq* polymerase. Buffer and cycling conditions were identical as above but nested PCR primers were used (supplementary table 1).

### Primer extension

To eliminate non-incorporated nucleotides, all nested PCR products of one blood sample were pooled and 5 µl of a 1:10 dilution of the pooled PCR products was digested with 2 U shrimp alkaline phosphatase (SAP) (Amersham Biosciences, Freiburg, Germany) in a reaction volume of 12 µl for 1 h at 37°C. SAP was inactivated by incubating samples for 15 min at 90°C.

Since most microarray scanners support only dual fluorescence measures simultaneously, a strategy of two parallel reactions had to be applied. Per sample, two primer extension reactions were carried out. The mixes differed in their combinations of Cy3 and Cy5 labelled ddNTPs (Perkin Elmer, Schwerzenbach, Switzerland) and extension primers were added as shown in supplementars table 2. It was thus possible to detect all possible SNP permutations in all loci using two fluorochromes only. All primer extension reactions for one sample were carried out in 2 x 20 µl containing 1 x Sequenase buffer, extension primer mix 1 or 2, and ddNTP mix 1 or 2, respectively (supplementary table 2), and 2 U Thermo Sequenase (Termipol<sup>®</sup>, Solis, Tartu, Estonia). Concentration of ddNTPs in both mixes was 0.25 µM and primers were diluted to a concentration of 6.25 nM each. The extension reaction was cycled 35 times with 94°C for 30 sec, and 50°C for 10 sec, with an initial cycle of 1 min at 94°C. After the extension reaction, both mixtures were pooled and 6 µl denaturing solution (3% SDS in 40 mM EDTA pH 8.0) were added. The sample was denatured at 95°C for 60 sec, and subsequently kept on ice until hybridisation onto the microarray.

## Chip production

Microarrays carried short oligonucleotides (20 - 35 bp) corresponding to the antisense DNA of the extension primers (supplementary table 1). All oligonucleotides possessed a C7-aminolinker and were spotted onto aldehyde activated glass slides (Genetix, Munich, Germany). Prior to spotting of oligonucleotides, a mask with 12 circular wells (diameter 8 mm) was applied onto the surface of the slides (MaProline GmbH, Starrkirch-Wil, Switzerland). Oligonucleotides were spotted in triplicates and pre-labelled Cy3 and Cy5 anchor oligonucleotides as well as four oligonucleotides with a random sequence were added as position and negative controls, respectively.

Slides were spotted using a VersArray ChipWriterPro system (Bio-Rad Laboratories, Hercules, CA). Oligonucleotides were dissolved in 180 mM phosphate buffer pH 8.0 and 0.5 nl of a 50  $\mu$ M solution were spotted onto the slides. Slides were stored desiccated and in the dark until used for hybridisation.

## Chip hybridisation

Twenty-three  $\mu$ l of the pooled and denatured primer extension reaction were transferred to a well of the microarray glass slide and 6  $\mu$ l of 20 x SSC was added. The hybridisation was carried out in a humid chamber at 50°C for 60 to 90 min. After hybridisation, the slide was washed at room temperature in 2 x SSC plus 2% SDS for 20 min, followed by another wash with 2 x SSC for 20 min, and a final wash with 2 x SSC plus 2% ethanol for 2 min. The slides were dried with compressed air and stored in the dark until scanned.

## Data acquisition

Hybridised slides were scanned at 635 nm and 532 nm using an Axon 4100A fluorescence scanner (Axon, Bucher Biotec AG, Basel, Switzerland). Cy3 and Cy5 images were acquired and analysed using the Axon GenePix<sup>®</sup> Pro (version 6.0) software ([www.axon.com](http://www.axon.com)). This software generates data points using pixel intensity after background subtraction. We developed a software for further analysis of raw data. Each signal was classified either as wild type, mutant, or mixed based on the expression intensities of the scanned image. The grouping was done according to the following algorithm: Fluorescence intensities below 9000 (Cy3) or 10000 (Cy5)

units (mean intensities minus background) were regarded as negative. For measures above these threshold values, we considered the ratio Cy5 to Cy3 intensity to discriminate wild-type, mutant, or mixed.

To determine an optimal algorithm to translate the output of the GenePix® Pro software into predictions about the genotypes present in analyzed samples, we used two singly infected blood samples which were previously sequenced at 29 SNP sites. Sequence data showed that the samples were different at 3 of 29 SNP sites (C59R, S108N, and A437G). The samples were analyzed with the chip either single or mixed in varying proportions (1:2, 1:4, 1:8, and 1:16). With this approach, we could empirically determine the following threshold values: for Cy5 to Cy3 ratios below 0.7 the sample was classified according to whether wild-type or mutant were labelled with Cy3. Ratios between 0.7 and 2.4 were assigned to mixed genotypes, and ratios above 2.4 to the Cy5 labelled genotype.

To estimate the above mentioned threshold parameters and to determine the predictive accuracy of our method, we used 3 of 4 identical but independently processed microarrays to estimate the threshold value to distinguish positive from negative signals so that the results would match the sequence data as close as possible. The fourth microarray was then used to apply this algorithm to determine the predictive accuracy of the method. This procedure was repeated 4 times in all possible combinations. Finally, we applied this algorithm to samples which were genotyped by PCR-RFLP and sequence analysis to determine the sensitivity and specificity of our method.

## Sequencing

PCR products were purified by size-selective polyethylenglycol precipitation (24) and directly sequenced using the respective nested PCR primers. Cycle sequencing (25 cycles of 96°C for 30 sec, 50°C for 15°C, and 60°C for 4 min) was performed using the API PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Schwerzenbach, Switzerland) and sequences were analysed using the API PRISM™ 310 Genetic Analyzer and the API PRISM™ software.

## RESULTS

### Amplification and sensitivity

Ten PCR fragments were required to cover all SNP locations. Amplicons sizes were 637 base pairs (bp) for *pfdhfr*, 686 bp for *pfdhps*, 799 bp and 526 bp for *pfmdr1*, five fragments of 630 bp, 548 bp, 476 bp, 304 bp, and 200 bp for *pfcr1*, and 798 bp for *pfATPase6*. To increase sensitivity we applied a nested PCR protocol and re-amplified all PCR products (for primers see supplementary table 1). For assay validation, DNA was extracted from parasitized erythrocytes added in ten fold dilutions to non-infected human blood. Thus, the amount of template per PCR reaction corresponded to 1 to 100'000 parasites. All samples were subjected to a primer extension reaction and hybridised onto the microarray. Figure 2 shows signals obtained from the dilution series at selected SNP sites. Except for *pfmdr1* 1246, we were able to detect 1000 parasites. Eight of 13 primers tested gave a signal with 10 parasites per reaction, and 6 primers were positive with 1 parasite only.

### Mixed infections

Since naturally occurring blood samples often contain multiple *P. falciparum* strains, we tested whether the presence of 2 different templates would decrease sensitivity of detection. We mixed 3D7 parasites and K1 parasites which differ in their genetic profile in *pfdhfr* 59 and 108. While one parasite strain was kept at 1% parasitaemia, the other strain was serially diluted from 4% to 0.00125%. When K1 was kept constant, 3D7 gave acceptable results even at the lowest dilutions, except with *dhfr* 59 with an endpoint at 0.0075%. When 3D7 was kept constant, similar results were obtained with decreasing template concentrations of K1. The exception was the loss of the K1 *pfdhfr* 59 signal at a dilution of 0.06%. Figure 3 shows the signals for both strains at both SNP positions. This experiment showed that the dynamic range for quantification of signals is low.

### Specificity

We have used 12 different culture strains from which we directly sequenced the genes *pfmdr1*, *pfcr1*, *pfdhps*, and *pfdhfr* comprising 16 different SNP sites. Of these

192 SNPs analysed, we failed to detect 1 SNP in 1 strain. A mixed signal was produced for 4 SNPs, and 7 SNPs gave discrepant results compared to sequencing, 3 of which at codon 86 of *pfmdr1*. This gave an overall specificity of 94% compared to the 'gold standard' of sequencing. After that, we determined the precision of base calling in naturally infected blood samples. We compared samples that were previously analysed by PCR-RFLP or sequencing with data from microarray analysis. Thirty-six PCR positive samples from field studies in Papua New Guinea were PCR-RFLP analyzed (25, 26, 27) for *pfmdr1* 86, *pfcr1* 76, *pfdhfr* 51, 59, and 108, and *pfdhps* 437, 540, and 581. Mean multiplicity of infection (MOI) in these samples, determined by genotyping the polymorphic *msp2* (merozoite surface protein 2) locus (28), was 1.56 (range 1-4, 95% CI = 1.29-1.84). Only 10 of these samples were positive by microscopy and densities were between 80 and 9440 asexual parasites per  $\mu$ l blood with a mean density of 1731/ $\mu$ l. Table 1 summarises the concordance between the microarray and PCR-RFLP analysis.

In addition, 12 of these samples harbouring a single clone infection were sequenced. There was an excellent agreement between the microarray and sequencing results (data not shown)

## Costs

Because we developed this microarray system to monitor parasite drug resistance against antimalarial drugs in resource restricted countries, it was essential to keep costs as low as possible so that the system can be used routinely for drug resistance monitoring. Cost calculation included consumables for DNA preparation, PCR reactions, primer extension with fluorochromes, and microarray production. But it does not take into account acquisition, maintenance and amortisation of equipment, nor does it take into account labour costs. We calculated a price of 0.27 EURO (0.33 US\$) per SNP when determining 32 SNP sites per sample and analysing 12 samples on one slide simultaneously.



## DISCUSSION

Monitoring of parasite resistance to antimalarial drugs has become an essential part of the malaria control programs in endemic countries. Common standards have been *in vivo* efficacy trials at health facilities (29) which are time and labour intensive. These studies are hampered, particularly in areas highly endemic for malaria, by the frequent re-occurrence of parasites from new inoculations. This leads to an underestimation of drug efficacy (2).

In order to circumvent these problems, systematic molecular monitoring of parasite resistance-associated SNPs has been widely promoted and used to complement *in vivo* efficacy studies (6, 7). However, current systems for SNP analysis are either extremely cumbersome and limited or expensive, both in terms of equipment and running costs.

Therefore, large studies analysing multiple SNPs of multiple genes in parallel have never been performed for reasons such as high costs and labour intensity. Here we report a novel method that allows the simultaneous analysis of many SNPs in hundreds of samples in a very short time (approx. 15h for 4 x 96 well plates) with significantly reduced costs. The microarray system was shown to be fast and accurate. In particular, the low detection limit of 10 to 100 parasites and the suitability for samples containing multiple infections represent added advantages over many competing systems. The significantly reduced costs per SNP compares favourably with other systems. In resource restricted countries, such as in Sub-Saharan Africa where parasite resistance to antimalarial drugs is a major concern (30), only a low cost system permits molecular monitoring of drug resistance.

In contrast to the analysis of diploid organisms, the analysis of *P. falciparum* infections represent an additional challenges because multiple infections are commonly found leading to a highly skewed distribution of different templates within a blood sample (31). In addition, PCR amplification might favour the dominant templates. Therefore, it was an important aim to ensure that minor template

populations can be detected. We therefore designed an elaborate algorithm to determine the detection threshold for genotype calling. But evidently, some low density infections may be missed in some individuals whatever threshold will be used. Whether this is important in the epidemiological assessments of resistance remains to be seen, because it is not clear to what degree these low density infections contribute to disease and transmission.

It has been shown that a synergistic action of transmembrane transporters is involved in parasite resistance to antimalarial drugs. In addition to *pfCRT*, another transporter involved in chloroquine resistance (*pfmdr1*), the homologue to the human P-glycoprotein, seems to contribute to resistance against chloroquine, the most commonly used drug against malaria (32). *Pfmdr1* has also been shown to modulate resistance to mefloquine and related drugs (33). But up to date, no clear association could be shown between individual SNPs and parasitological failure of a given drug. Hence, it is possible that the parallel analysis of all SNPs in several genes might identify certain haplotypes suspected to be involved in drug resistance. With the prospect of analysing all known drug resistance associated SNPs at once, elucidation of the genetic background of drug failure becomes feasible. This underscores the need for linking individual SNPs into haplotypes because interactions between SNPs from different loci are likely to account for the phenotypic effect. However, current algorithms and techniques are yet unable to generate true haplotypes of unlinked loci in samples containing multiple infections of *P. falciparum*. In Tanzania for instance, mean multiplicity of infection (MOI) in children is 5 concurrent infections per individual (34), considerably complicating or preventing the determination of haplotypes of individual *P. falciparum* clones. The ability of our method to semi-quantify signal strength potentially allows the determination of the most dominant haplotype. Since parasite density is a correlate of malaria symptoms, the most dominant haplotype within a multiple clone infection is likely to represent the clone actually causing clinical malaria.

We have now used our microarray system successfully for drug resistance monitoring in several sites over three years, in Tanzania (23), Papua New Guinea (22), and Solomon islands (unpublished). This demonstrates that standardised and

comparable data can be produced at an affordable price. The flexibility of the system facilitates prompt inclusion of newly identified point mutations associated with parasite resistance.

In conclusion, this method offers unmatched capacity to provide evidence-based data on the dynamics of parasite resistance against antimalarial drugs in a cost-effective way. This platform can also be widely applied and adapted with ease to other genotyping tasks requiring highly parallel multiple SNP analyses.

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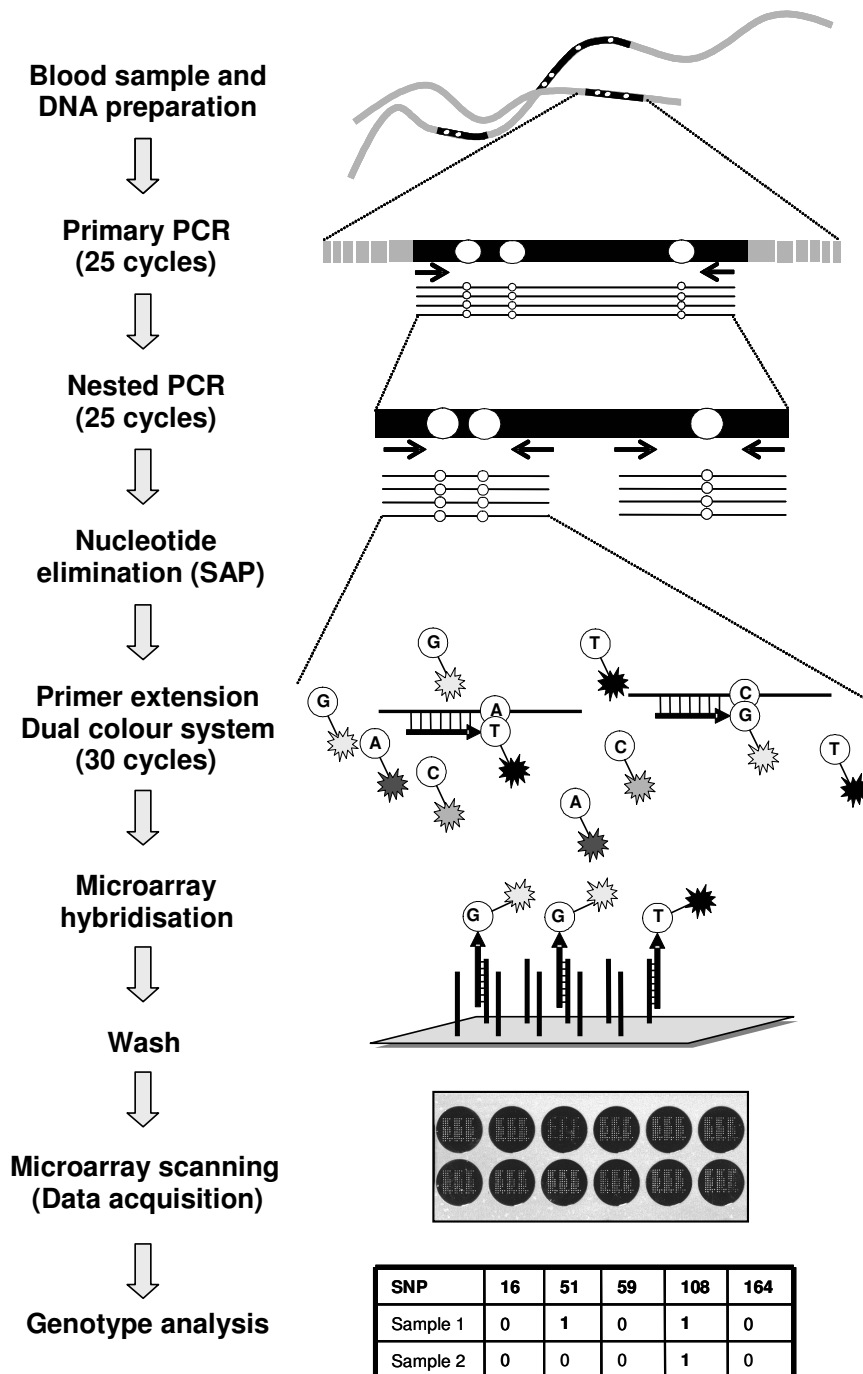
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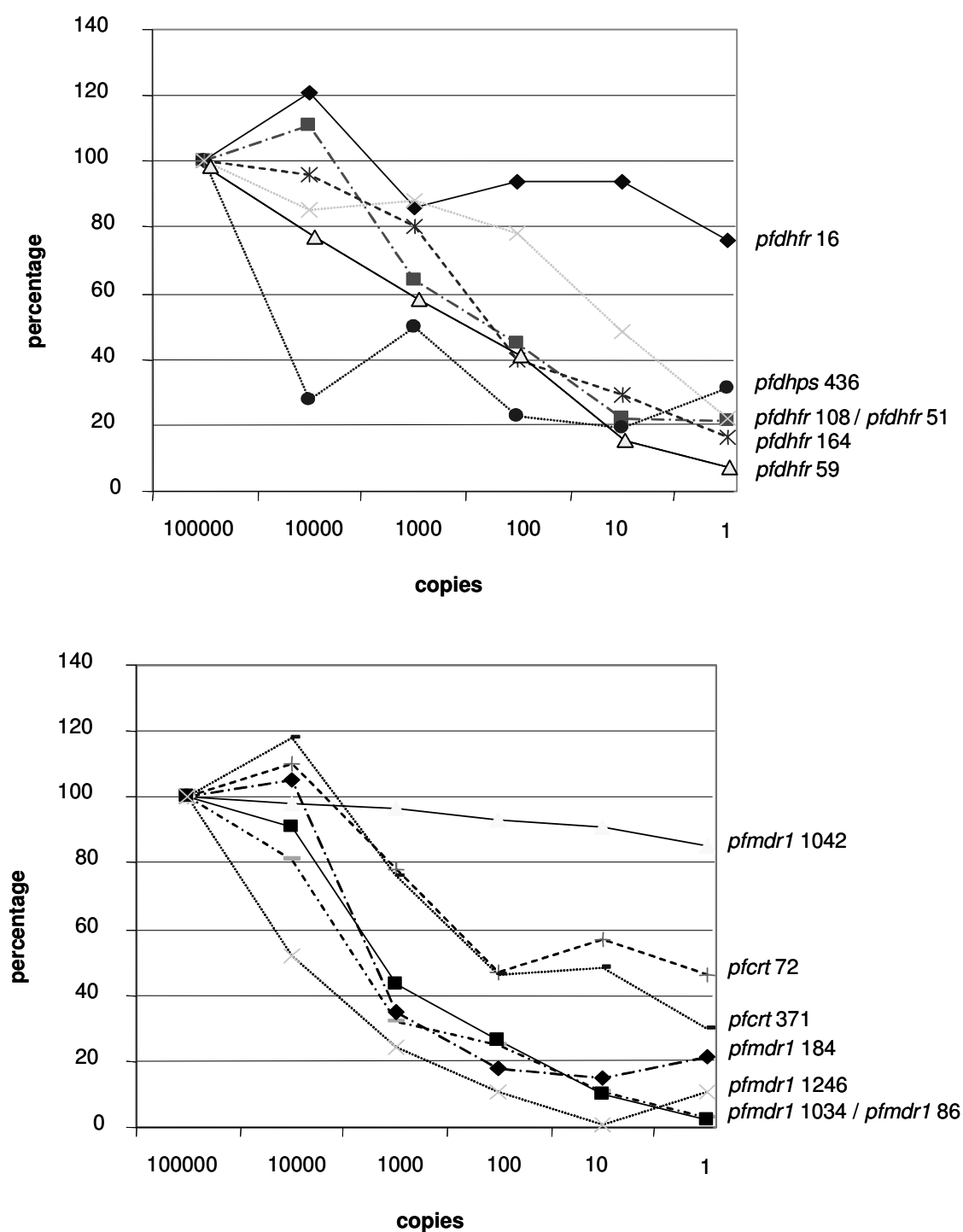
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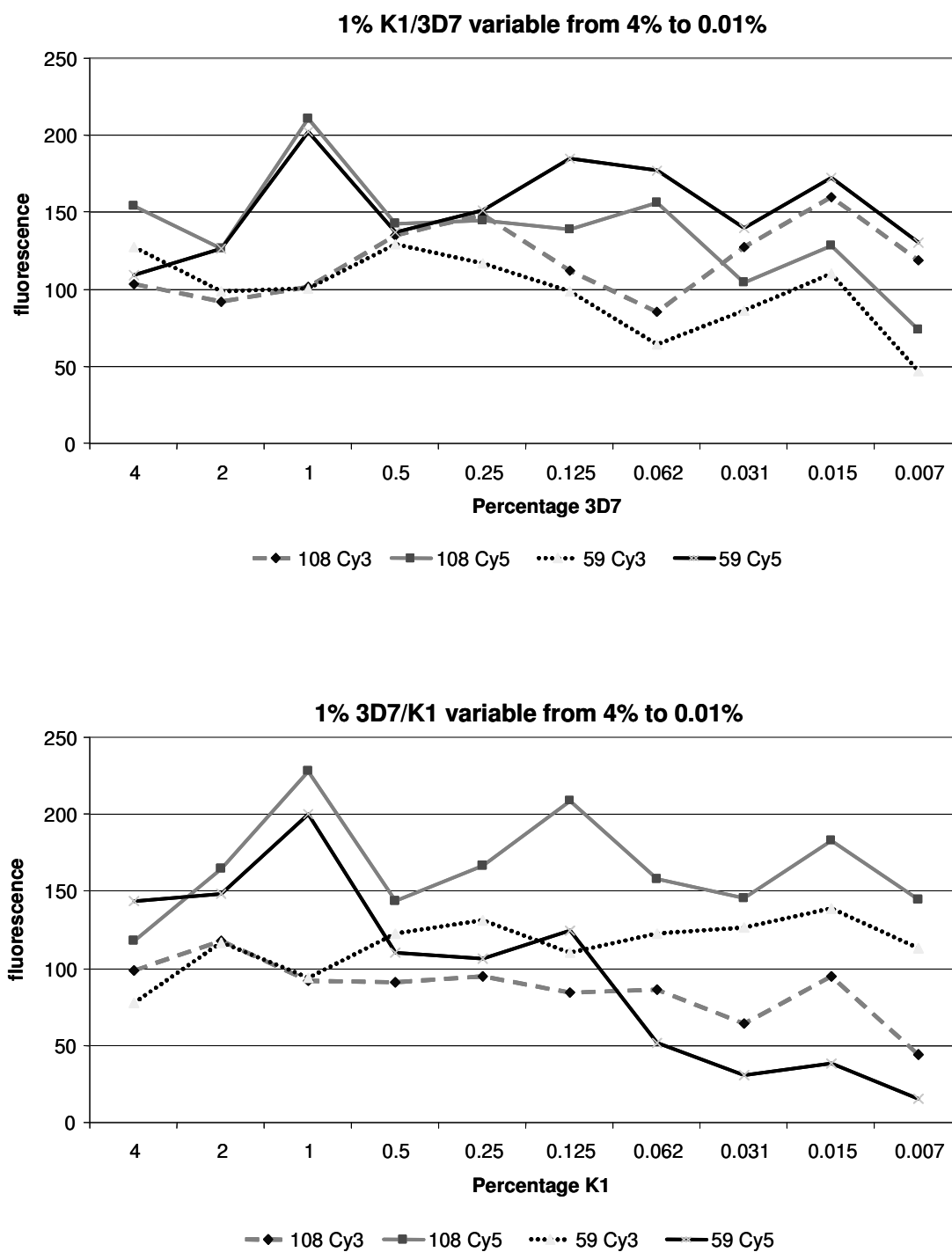


**Figure 1:** Flow diagram of analytical procedure starting from blood samples collected in the field. DNA is prepared from blood samples and target sequences amplified by nested PCR. All amplicons are subsequently combined and nucleotides are eliminated by SAP. Primer extension is performed in 2 x 20 µl for each sample and both mixtures are combined for hybridisation onto the microarray. After washing, the array is air dried and scanned and subsequently analysed using GenePix® Pro and dedicated analysis software.





**Figure 2:** Sensitivity curves for SNP analysis in parasite samples diluted in uninfected blood. All data represent the percentage of fluorescence of the undiluted sample containing the genomic equivalent of 100000 parasites per reaction. The upper panel represents values obtained for SNPs within the *pfdhfr* and *pfdhps* locus. The lower panel represents the values for *pfmdr1* and *pfcr1*.



**Figure 3:** Detection of SNPs in mixed parasite infections. The upper panel depicts arbitrary fluorescence values obtained when strain K1 was mixed with various dilutions of the 3D7 strain. K1 and 3D7 differ at codons 59 and 108 in the *pfdhfr* gene. The lower panel shows arbitrary fluorescence values obtained when strain 3D7 was mixed with various dilutions of the K1 strain.

**Table 1:** SNP analysis in 36 field samples from Papua New Guinea and agreement between data obtained by microarray and PCR-RFLP method, respectively

	N	nd RFLP	nd array	Concordant	mixed RFLP/ single array*	mixed array/ single RFLP	single RFLP / single RFLP alternative nt	% agreement ( $\kappa$ test)
Locus	36	n=1	n=0	250 nt 86.8%	25 nt 8.3%	8 nt 2.8%	5 nt 1.7%	Total: 288 nt
<i>mdr1</i> 86	36	0	0	36	0	0	0	100
<i>crt</i> 76	35	1	0	28	6	0	1	80
<i>dhfr</i> 51	36	0	0	23	13	0	0	63.9
<i>dhfr</i> 59	36	0	0	29	4	2	1	80.6
<i>dhfr</i> 108	36	0	0	29	0	6	1	80.6
<i>dhps</i> 437	36	0	0	34	1	0	1	94.4
<i>dhps</i> 540	36	0	0	35	0	0	1	97.2
<i>dhps</i> 581	36	0	0	36	0	0	0	100

N, total number of samples

nd, not determined

\* identification of mutant or wild type alleles only requires complete digest

nt, nucleotides

n, number of samples

**Supplementary table 1:** Oligonucleotide primers used (Operon Biotechnologies GmbH, Cologne, Germany)

Name	Locus / fragment	Sequence
	Primary PCR amplification	5' 3'
<b>P5-for</b>	<i>dhfr</i>	TTTATGATGGAACAAGTCTGC
<b>P5-1 rev</b>	<i>dhfr</i>	ATTCATATGTACTATTTATTCTAGT
<b>P8-1 for</b>	<i>dhps</i>	ATTTTGTGTAACCTAAACGTGCTGTTCA
<b>P8-1 rev</b>	<i>dhps</i>	CTTGTCTTTCCTCATGTAATTCATCT
<b>P1-1 for</b>	<i>mdr1</i> , first fragment	TTAAATGTTTACCTGCACAACATAGAAAATT
<b>P1-1 rev</b>	<i>mdr1</i> , first fragment	CTCCACAATAACTTGCAACAGTTCTTA
<b>P3-1 for</b>	<i>mdr1</i> , second fragment	AATTTGATAGAAAAAGCTATTGATTATAA
<b>P3-1 rev</b>	<i>mdr1</i> , second fragment	TATTTGGTAATGATTGATAAAATTCATC
<b>P10-1 for</b>	<i>crt</i> , first fragment	TTGTCGACCTTAACAGATGGCTCAC
<b>P10-1 rev</b>	<i>crt</i> , first fragment	AATTTCCCTTTTTATTTCCAAATAAGGA
<b>P18-1 for</b>	<i>crt</i> , second fragment	ACTTTATTTGTATGATTATGTTC
<b>P18-1 rev</b>	<i>crt</i> , second fragment	TAACTGCTCCGAGATAATTGT
<b>P11-1 for</b>	<i>crt</i> , third fragment	ATTTACTCCTTTTTAGATATCACTTA
<b>P11-1 rev</b>	<i>crt</i> , third fragment	TTATATTTTTTAAAACTATTTCCCTTG
<b>P16-1 for</b>	<i>crt</i> , fourth fragment	TCTGTTATTTTTATTTCTTATAGGCTAT
<b>P16-1 rev</b>	<i>crt</i> , fourth fragment	CTTGTATGTATCAACGTTTTTCATCC
<b>P12-1 for</b>	<i>crt</i> , fifth fragment	AGGAAATAAATATGGAATGTTTAATTGA
<b>P12-1 rev</b>	<i>crt</i> , fifth fragment	TTCTAAGATAATATTTCTACACGGT
<b>P17-1 for</b>	<i>ATPase6</i>	AATATTGTTATTCAGAATATGATTATAA
<b>P17-1 rev</b>	<i>ATPase6</i>	TGGATCAATAATACCTAATCCACCTA
	Nested PCR amplification	5' 3'
<b>P5 for</b>	<i>dhfr</i>	ACAAGTCTGCGACGTTTTTCGATATTTATG
<b>P5 rev</b>	<i>dhfr</i>	AGTATATACATCGCTAACAGA
<b>P8 for</b>	<i>dhps</i>	TTGAAATGATAAATGAAGGTGCTAGT
<b>P8 rev</b>	<i>dhps</i>	CCAATTGTGTGATTTGTCCA
<b>P1 for</b>	<i>mdr1</i> , first fragment	TGTATGTGCTGTATTATCAGGA
<b>P1 rev</b>	<i>mdr1</i> , first fragment	CTCTTCTATAATGGACATGGTA
<b>P3 for</b>	<i>mdr1</i> , second fragment	GAATTATTGTAAATGCAGCTTTA
<b>P3 rev</b>	<i>mdr1</i> , second fragment	GCAGCAAACCTTACTAACACG
<b>P10 for</b>	<i>crt</i> , first fragment	CTTGTCTTGGTAAATGTGCTC
<b>P10 rev</b>	<i>crt</i> , first fragment	GAACATAATCATACAAATAAAGT
<b>P18 for</b>	<i>crt</i> , second fragment	TCCTTATTTGGAAATAAAAAGGGAAATT
<b>P18 rev</b>	<i>crt</i> , second fragment	TAAGTGATATCTAAAAAGGAGTAAAT

<b>P11 for</b>	<i>crt</i> , third fragment	ACAATTATCTCGGAGCAGTTA
<b>P11 rev</b>	<i>crt</i> , third fragment	CATGTTTGAAAAGCATACAGGC
<b>P16 for</b>	<i>crt</i> , fourth fragment	CTTTTCCAATTGTTCACTTCTTG
<b>P16 rev</b>	<i>crt</i> , fourth fragment	TCTTACATAGCTGGTTATTAAAT
<b>P12 for</b>	<i>crt</i> , fifth fragment	ACCATGACATATACTATTGTTAG
<b>P12 rev</b>	<i>crt</i> , fifth fragment	TTATAGAACCAAATAGGTAGCC
<b>P17 for</b>	<i>ATPase6</i>	AGCAAATATTTTCTGTAACGATAATA
<b>P17 rev</b>	<i>ATPase6</i>	TGTTCTAATTTATAATAATCATCTGT
	Extension primer at SNP site	5' 3'
<b>16</b>	<i>dhfr</i> 16	GACGTTTTTCGATATTTATGCCATATGTG
<b>51</b>	<i>dhfr</i> 51	GAAATAAAGGAGTATTACCATGGAAATGTA
<b>59</b>	<i>dhfr</i> 59	TTCACATATGTTGTAACCTGCAC
<b>108</b>	<i>dhfr</i> 108 forward	CAAAATGTTGTAGTTATGGGAAGAACAA
<b>108B</b>	<i>dhfr</i> reverse	AAAGGTTTAAATTTTTTGAATGCTTCCCAG
<b>164</b>	<i>dhfr</i> 164 forward	GGGAAATTAAATTACTATAAATGTTTTATT
<b>164B</b>	<i>dhfr</i> 164 reverse	TTCTTGATAAACAACGGAACCTCCTA
<b>436</b>	<i>dhps</i> 436	TTATAGATATAGGTGGAGAATCC
<b>437</b>	<i>dhps</i> 437 reverse	TTGGATTAGGTATAACAAAAGGA
<b>540</b>	<i>dhps</i> 540	AGGAAATCCACATACAATGGAT
<b>581</b>	<i>dhps</i> 581	GGATACTATTTGATATTGGATTAGGATTTG
<b>613</b>	<i>dhps</i> 613 forward	GGATATTCAAGAAAAAGATTTATT
<b>613B</b>	<i>dhps</i> 613 reverse	ATTTTGATCATTTCATGCAATGGG
<b>640</b>	<i>dhps</i> 640 reverse	CAATTGTGTGATTTGTCCACAA
<b>645</b>	<i>dhps</i> 645	ATAAAAATATTGTGGACAAATCAC
<b>86</b>	<i>mdr1</i> 86	TTTGGTGAATATTAAGAACATG
<b>184</b>	<i>mdr1</i> 184	TGCCAGTTCCTTTTTAGGTTTAT
<b>1034</b>	<i>mdr1</i> 1034	ATTGTAAATGCAGCTTTATGGGGATTC
<b>1042</b>	<i>mdr1</i> 1042 reverse	AGAAGGATCCAAACCAATAGGCAAACTAT
<b>1246</b>	<i>mdr1</i> 1246	TAATATATGTGATTATAACTTAAGA
<b>72</b>	<i>crt</i> 72	TTTTAAGTATTATTTATTTAAGTGTA
<b>76</b>	<i>crt</i> 76 reverse	TTTGTTTAAAGTTCTTTTAGCAAAATT
<b>97</b>	<i>crt</i> 97	GTTTTGTAACATCCGAAACTCA
<b>152</b>	<i>crt</i> 152	CCTTCATAGGTCTTACAAGAACT
<b>163</b>	<i>crt</i> 163	ATCCAATCATTTGTTCTTCAATTAAG
<b>220</b>	<i>crt</i> 220	TTCTATCATATTTAATCTTGCTTAATTAGT
<b>271</b>	<i>crt</i> 271	TATACACCCTTCCATTTTTAAAA
<b>326</b>	<i>crt</i> 326	AAACCTTCGCATTGTTTCCTTCTTT
<b>326B</b>	<i>crt</i> 326 reverse	ACATAGCTGGTTATTAAATTATCACAAATG
<b>356</b>	<i>crt</i> 356	TTGTTAGTTGTATACAAGGTCCAGCA
<b>356B</b>	<i>crt</i> 356 reverse	GGCTAAGAATTTAAAGTAATAAGCAATTGCT

<b>371</b>	<i>crt</i> 371	CTTTTAAATTTTATAGGGTGATGTTGTAA
<b>538</b>	<i>ATPase6</i> 538	AAATGTAATAAAGCTAATTCGGT
<b>574</b>	<i>ATPase6</i> 574	TGAAAAAATACAACACCTGTAC
<b>623</b>	<i>ATPase6</i> 623	AACCATTCTAATTATACTACAGCTCAGG
<b>683</b>	<i>ATPase6</i> 683	TGAATGTATTTCTTCTTGGAGAAA
<b>769</b>	<i>ATPase6</i> 769	ACTTAGCTTTGCTTATAAAAAATTAA
	Arrayed as antisense oligonucleotides	5' 3' ---- C7
<b>16 C-7</b>	<i>dhfr</i> 16	CACATATGGCATAAATATCGAAAACGTC
<b>51 C-7</b>	<i>dhfr</i> 51	TACATTTCCATGGTAATACTCCTTTATTTTC
<b>59 C-7</b>	<i>dhfr</i> 59	GTGCAGTTACAACATATGTGAA
<b>108 C-7</b>	<i>dhfr</i> 108 forward	TTGTTCTTCCATAACTACAACATTTTG
<b>108B C-7</b>	<i>dhfr</i> reverse	CTGGGAAAGCATTCCAAAAAATTTAAACCTTT
<b>164 C-7</b>	<i>dhfr</i> 164 forward	AATAAAACATTTATAGTAATTTAATTTCCC
<b>164B C-7</b>	<i>dhfr</i> 164 reverse	TAGGAGGTTCCGTTGTTTATCAAGAA
<b>436 C-7</b>	<i>dhps</i> 436	GGATTCTCCACCTATATCTATAA
<b>437 C-7</b>	<i>dhps</i> 437 reverse	TCCTTTTGTTATACCTAATCCAA
<b>540 C-7</b>	<i>dhps</i> 540	ATCCATTGTATGTGGATTTCCA
<b>581 C-7</b>	<i>dhps</i> 581	CAAATCCTAATCCAATATCAAATAGTATCC
<b>613 C-7</b>	<i>dhps</i> 613 forward	AATAAATCTTTTTCTTGAATATCC
<b>613B C-7</b>	<i>dhps</i> 613 reverse	CCCATTGCATGAATGATCAAAAT
<b>640 C-7</b>	<i>dhps</i> 640 reverse	TTGTGGACAAATCACACAATTG
<b>645 C-7</b>	<i>dhps</i> 645	GTGATTTGTCCACAATATTTTTAT
<b>86 C-7</b>	<i>mdr1</i> 86	CATGTTCTTTAATATTACACCAAA
<b>184 C-7</b>	<i>mdr1</i> 184	ATAAACCTAAAAAGGAAGTGGCA
<b>1034 C-7</b>	<i>mdr1</i> 1034	GAATCCCATAAAGCTGCATTTACAAT
<b>1042 C-7</b>	<i>mdr1</i> 1042 reverse	ATAGTTTTGCCTATTGGTTTGGATCCTTCT
<b>1246 C-7</b>	<i>mdr1</i> 1246	TCTTAAGTTATAATCACATATATTA
<b>72 C-7</b>	<i>crt</i> 72	TACACTTAAATAAATAATACTTAAAA
<b>76 C-7</b>	<i>crt</i> 76 reverse	AATTTTTGCTAAAAGAAGCTTTAAACAAA
<b>97 C-7</b>	<i>crt</i> 97	TGAGTTTCGGATGTTACAAAAC
<b>152 C-7</b>	<i>crt</i> 152	AGTTCTTGTAAGACCTATGAAGG
<b>163 C-7</b>	<i>crt</i> 163	CTTAATTGAAGAACAAATGATTGGAT
<b>220 C-7</b>	<i>crt</i> 220	ACTAATTAAGACAAGATTAAATATGATAGAA
<b>271 C-7</b>	<i>crt</i> 271	TTTTAAAAATGGAAGGGTGTATA
<b>326 C-7</b>	<i>crt</i> 326	AAAGAAGGAAAACAATGCGAAGGTTT
<b>326B C-7</b>	<i>crt</i> 326 reverse	CATTTGTGATAATTTAATAACCAGCTATGT
<b>356 C-7</b>	<i>crt</i> 356	TGCTGGACCTTGTATACAATAACAA
<b>356B C-7</b>	<i>crt</i> 356 reverse	AGCAATTGCTTATTACTTTAAATTCTTAGCC
<b>371 C-7</b>	<i>crt</i> 371	TTACAACATCACCTATAAAATTAAAAAG
<b>538</b>	<i>ATPase6</i> 538	ACCGAATTAGCTTTATTACATTT

<b>574</b>	<i>ATPase6</i> 574	GTACAGGTGTTGTATTTTTTTCA
<b>623</b>	<i>ATPase6</i> 623	CCTGAGCTGTAGTATAATTAGAATGGTT
<b>683</b>	<i>ATPase6</i> 683	TTTCTCCAAGAAGAAATACATTCA
<b>769</b>	<i>ATPase6</i> 769	TTAATTTTTTATAAGCAAAGCTAAGT

**Supplementary table 2:** ddNTP and extension primer mixes Combination 1 and Combination 2

ddNTP mix	Combination 1	Combination2
	ddATP <b>Cy3</b>	ddUTP <b>Cy3</b>
	ddCTP <b>Cy3</b>	ddCTP <b>Cy3</b>
	ddGTP <b>Cy5</b>	ddATP <b>Cy5</b>
	ddUTP <b>Cy5</b>	ddGTP <b>Cy5</b>
Extension primer mixes	Combination 1	Combination2
<i>Pfdhps</i>	437, 540, 581, 613, 640	436, 613B, 645
<i>Pfdhfr</i>	16, 51, 59, 108, 164	108B, 164B
<i>Pfmdr1</i>	86, 184, 1034, 1042	1246
<i>Pfcrt</i>	72, 152, 271, 326, 326B, 356, 356B	76, 97, 163, 220, 371
<i>PfATPase6</i>	538, 769	574, 623, 683





## Chapter 4

### **The usefulness of twenty-four molecular markers in predicting treatment outcome with combination therapy of amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against falciparum malaria in Papua New Guinea**

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## ABSTRACT

**BACKGROUND** In Papua New Guinea (PNG), combination therapy with amodiaquine (AQ) or chloroquine (CQ) plus sulphadoxine-pyrimethamine (SP) was introduced as first-line treatment against uncomplicated malaria in 2000.

**METHODS** The aim of the study was to characterise 24 molecular markers of drug resistance in pre-treatment samples collected in two different areas in PNG and to investigate the association between infecting genotype and treatment response in order to identify useful predictors of treatment failure with combination therapy.

**RESULTS** In 2004, overall failure rate up to Day 28 for *P. falciparum* malaria was 28% in the Karimui and 16% in the Wosera area. The strongest independent predictors for treatment failure with AQ or CQ plus SP were *pfmdr1* N86Y (OR=9.26,  $p<0.01$ ) and *pfdhps* A437G (OR=3.82,  $p<0.01$ ). Mutations found in CQ related markers *pfcr1* K76T, A220S, N326D, and I356L did not help to increase the predictive value, the most likely reason being that these mutations reached almost fixed levels. Though mutations in SP related markers *pfdhfr* S108N and C59R were not associated with treatment failure, they increased the predictive value of *pfdhps* A437G. The difference in clinical outcome was reflected in the corresponding genetic profile of the parasite populations in the two sites, with significant differences seen in the frequencies of mutant *pfmdr1* N86Y, *pfcr1* A220S, and *pfdhps* A437G.

**CONCLUSION** The study provides evidence for high levels of resistance to the combination regimen of AQ or CQ plus SP in Papua New Guinea and indicates which of the many molecular markers analysed are useful for the monitoring of parasite resistance to combinations with AQ or CQ plus SP.

## INTRODUCTION

The effectiveness of the most widely used first-line antimalarials chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) has been heavily compromised by the emergence and spread of *P. falciparum* resistance to these drugs. In order to improve treatment efficacy and to delay the development and spread of drug resistance, there is strong advocacy for combination therapy (White & Olliaro, 1996). Though many authorities recommend the combination of 4-aminoquinolines or SP with artemisinin derivatives (WHO, 2001) this option is expensive and several countries have taken an interim step and chose the inexpensive combination of amodiaquine (AQ) or CQ plus SP.

Monitoring of parasite resistance is essential in directing the rational use of antimalarials. Apart from studies assessing *in vivo* drug efficacy and *in vitro* drug sensitivity, molecular markers have been proposed as a means to monitor drug resistant malaria (WHO, 2003).

CQ resistance has been attributed to several mutations occurring in the *Plasmodium falciparum* chloroquine transporter gene (*pfcr*) and *Plasmodium falciparum* multidrug resistance gene 1 (*pfmdr1*), both encoding proteins localised in the digestive vacuole of the parasite (Fidock *et al.*, 2000; Reed *et al.*, 2000; Sidhu *et al.*, 2002). Correlation between molecular markers of CQ resistance and *in vivo* treatment outcome has been complex. Whereas several studies have shown the key role of *pfcr* K76T in conferring *in vivo* resistance to CQ (Babiker *et al.*, 2001; Djimdé *et al.*, 2001; Wellems & Plowe, 2001), the relationship between phenotypic resistance and other *pfcr* polymorphisms (i.e., C72S/R, M74I/T, N75E/D/K/I, K76T/I/N, I77T, H97Q/L, A144F/T, L148I, L160Y, I194T, A220S, Q271E, N326S/D, I356V/T/L and R371T/I), which have been shown to be associated with CQ resistance *in vitro* (reviewed in Cooper *et al.*, 2005), has been poorly studied in the field. Single-base changes in *pfmdr1* N86Y, Y184F, S1034C, N1042D and D1246Y have been documented in CQ resistant laboratory strains, but a straightforward association of these polymorphisms with *in vivo* CQ resistance has been questioned by several studies (Basco & Ringwald, 1998; Haruki *et al.*, 1994; Pillai *et al.*, 2001; Pova *et al.*, 1998).

The accumulation of point mutations in *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*), two enzymes in the parasite's folate synthesis pathway, is associated with resistance to SP (Cowman, *et al.*, 1988; Peterson *et al.*, 1988; Triglia *et al.*, 1997; Triglia *et al.*, 1998). Though the relationship between polymorphisms in these genes and resistance to SP has been shown *in vitro*, the correlation of different

genotypes and clinical treatment outcome is controversial. Whereas the triple mutation S108N+C59R+N51I in *pfdhfr* has been found to be a good molecular marker for SP resistance by some authors (Basco *et al.*, 1998; Basco *et al.*, 2000; Wang *et al.*, 1997), others did not confirm the usefulness of this combination of mutations (Alifrangis *et al.*, 2003; Aubouy *et al.*, 2003). The quintuple mutation *pfdhfr* S108N+C59R+N51I plus *pfdhps* A437G+K540E has been proposed as a useful indicator for monitoring SP resistance in Africa (Nzila, *et al.*, 2000), in the Amazon region, the quintuple mutation *pfdhfr* S108N+N51I+I164L plus *pfdhps* A437G+K540E has been shown to be more useful (Kublin *et al.*, 1998). More recently, several authors have found the double mutation *pfdhfr* C59R plus *pfdhps* K540E to be sufficient to predict treatment failure *in vivo* (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003; Talisuna *et al.*, 2004). The most likely reason for these conflicting reports is the fact that, apart from the infecting genotype, response to drug treatment is affected by many factors, such as host immunity, which is related to transmission intensity, and history of drug use in a given area (Alifrangis *et al.*, 2003; Omar *et al.*, 2001; Staedke *et al.*, 2004). As a consequence, the patterns as well as the predictive values of molecular drug resistance markers may vary between different geographical regions. Another problem is that most of the studies looked at only few markers, which does not allow investigating which of the many known markers are the most useful for parasite resistance monitoring to specific drugs.

After a long history of 4-aminoquinoline use which has been accompanied by accumulating reports about increasing levels of AQ and CQ resistance (Müller *et al.*, 2003; Genton *et al.*, 2006), official drug policy for uncomplicated malaria in Papua New Guinea (PNG) was changed to the combination therapy of AQ or CQ plus SP in 2000. Although high levels of polymorphisms in CQ relevant genes *pfcr*t and *pfmdr*1, and also to a lesser extent in key markers responsible for resistance to SP, have already been reported in PNG (Casey, *et al.*, 2004; Mehlotra *et al.*, 2001; Reeder *et al.*, 1996;), their association with *in vivo* treatment outcome has never been evaluated.

In this study, we analysed the genetic profile of parasites collected from pre-treatment samples of malaria patients attending two health facilities in PNG with known clinical and parasitological outcomes after treatment with AQ or CQ plus SP. Twenty-four key markers in *pfmdr*1, *pfcr*t, *pfdhfr* and *pfdhps* were determined using a new DNA microarray-based technology. The relation of the parasite genetic output to the treatment response was investigated to identify the most useful predictors of failure with the current first-line regimen in the country.

## MATERIALS AND METHODS

### *In vivo* assessment of drug efficacy

Drug efficacy studies were conducted according to the standardised WHO protocol for low to moderate transmission areas (WHO, 2003) and are described in detail elsewhere (Marfurt *et al.*, 2006, submitted, Chapter 2). Children between 6 months and 7 years of age were enrolled if they were presenting at the health centre with clinically overt and microscopically confirmed *P. falciparum* malaria and no danger signs for severe or complicated malaria (WHO, 2000) or signs of any other disease, malnutrition or anaemia. Standard AQ (for patients <14 kg) or CQ plus SP first line treatment (10 mg AQ or CQ per kg on Day 0, 1 and 2, and 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 0) was administered under supervision over the first three days. Visits for the follow-up were scheduled on Day 1, 2, 3, 7, 14, and 28. On every visit, patients were clinically examined and a Giemsa-stained blood slide was taken for the microscopic assessment of parasitaemia. A blood sample was taken on Day 0 (pre-treatment sample) and on Days 14 and 28 or any day of treatment failure for molecular genotyping purposes. At the end of the follow-up, the patients were classified according to their clinical and parasitological responses into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR) (WHO, 2003).

### Study sites and population

The studies were conducted between October 2003 and April 2004 in the Karimui area (Simbu Province) and the South Wosera area (East Sepik Province), two rural places mesoendemic for malaria but differing with regard to transmission intensity and drug use patterns (Müller *et al.*, 2003). Main characteristics of the study populations and the two sites are depicted in table 1.

Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG and consent was obtained from parents or legal guardians prior to recruitment of each patient.

## Laboratory analyses

DNA was extracted using QIAamp<sup>®</sup> DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

Assessment of single nucleotide polymorphisms (SNPs) for drug resistant malaria was done for *pfmdr1* codons N86Y, Y184F, S1034C, N1042D and D1246Y, *pfcr1* codons K76T, H97Q, T152A, S163R, A220S, Q271E, N326D/S, I356L/T and R371I, *pfdhfr* codons A16V, N51I, C59R, S108N/T and I164L, and *pfdhps* codons S436A, A437G, K540E, A581G, and A613T/S. The method is based on parallel PCR amplification of the target sequences followed by primer extension mediated mini-sequencing using fluorochrome-labelled ddNTPs. Subsequent base calling occurs on a microarray upon sequence specific hybridisation (Cramer *et al.*, 2006, submitted, Chapter 3).

Assessment of the multiplicity of infection (MOI) in pre-treatment samples and the differentiation between true recrudescences and new infections in treatment failure samples was done by PCR-RFLP analysis of the merozoite surface protein 2 (*msp2*) as previously described (Cattamanchi *et al.*, 2003; Felger and Beck, 2002; Slater *et al.*, 2005).

## Statistics

Statistical analyses were performed by the use of STATA software (version 8.2; Stata Corp., College Station, Texas). The strength of association was evaluated by calculating odds ratios (OR). We used  $\chi^2$  tests and Fisher's exact test and stepwise logistical regression analysis as applicable to assess the significance of association between known risk factors and single or multiple mutations and treatment failure.

To estimate the allele frequencies of resistance markers in our sample set, we used a non linear statistical model that takes into account the effects of varying multiplicity of infection and assumes that resistant and sensitive parasite clones are transmitted independently. The likelihood of a sample containing no resistant clones is  $(1 - p)^n$ , where  $p$  is the frequency for the mutant allele and  $n$  is the multiplicity of infection of the sample. Similarly, the likelihood for the sample to contain no wild-type allele is  $p^n$  and for a mixture of both, a wild-type and a resistant allele, is  $1 - p^n - (1 - p)^n$ . The likelihood over the whole data set for  $p$  is computed as the product of this likelihood over all samples, using values of  $n$  derived from *msp2* genotyping results. A Markov Chain Monte Carlo algorithm (Program Winbugs 1.3) was used

to obtain estimates of mutant allele frequencies and credible intervals (Bayesian confidence intervals (CI) for  $p$ ), making use of this likelihood, and assuming a uniform (0.1) prior distribution for  $p$  (Schneider *et al.*, 2002).

## RESULTS

### *In vivo* drug efficacy

A total of 97 patients in Karimui and 112 patients in the Wosera were enrolled into the study and treated with AQ plus SP (174/209 (83.25%), median age of 4 years) or CQ plus SP (35/209 (16.75%), median age of 6.5 years). Overall treatment failure rate up to Day 28 for *P. falciparum* was 31% in Karimui and 20% in the Wosera. Three of 28 (10.7%) late treatment failure cases in Karimui and 4 of 17 (23.5%) in the Wosera were new infections as shown by molecular genotyping of the highly polymorphic *msp2* locus. Therefore, overall treatment failure rates after PCR-correction, classifying infections with new and recurrent strains as true recrudescences (i.e., treatment failures), were 28% in Karimui and 16% in the Wosera area (Table 2).

### Prevalence and relationship of *pfmdr1*, *pfprt*, *pfdhfr* and *pfdhps* mutations

Mutation analyses were successfully accomplished in 206 (99%) of all pre-treatment samples from both study sites. Polymorphisms were found in *pfmdr1* codons N86Y, Y184F, and N1042D, *pfprt* codons K76T, A220S, N326D and I356L, *pfdhfr* codons C59R and S108N, and *pfdhps* codons A437G and K540E. None of the other SNPs (11/24) was detected as mutated allele in any of the infections analysed. Regarding CQ relevant molecular markers, infections harbouring mutated *pfmdr1* N86Y and *pfprt* K76T, N326D, I356L and A220S alleles were with 86%, 91%, 89%, 89%, and 70% very common, whereas 5%, 1%, 1%, 0%, and 2% of these infections were mixed with a wild-type allele (Figure 1). The mutated alleles in *pfmdr1* Y184F and *pfmdr1* N1042D were only found in 5 (2%) and 2 (1%) samples, respectively, with the latter being detected as mixed allele only.

Considering relationships of mutated alleles in *pfprt*, we found that 1) the mutations N326D and I356L were always linked, 2) the double mutation N326D+I356L never occurred without a mutated allele K76T, and 3) a mutation A220S never occurred without the triple mutation K76T+N326D+I356L. Considering *pfmdr1*, a mutation N1042D was always linked to a mutated Y184F allele, but we never observed these mutated alleles occurring together with a N86Y mutation.



Regarding SP relevant molecular markers, mutations in *pfdhfr* S108N and C59R were also very common with 79% and 77% of infections having a pure mutant, and 91% and 82% of infections having a mutant or mixed allele, respectively. Mutated alleles in *pfdhps* A437G were found in 13% of all infections whereas in 10% of all infections it was detected as a pure mutant. The *pfdhps* K540E mutation was only found in 2 (1%) samples and was only detected as pure mutant allele. *Pfdhfr* C59R was never detected without *pfdhfr* S108N, and *pfdhps* A437G was strongly linked to the double mutation *pfdhfr* S108N+C59R with only 2 (7.6%) of the samples having the mutant allele without any mutated allele in *pfdhfr*. Also the *pfdhps* K540E mutation was only found in conjunction with the double mutation *pfdhfr* S108N+C59R.

#### **Association between *pfmdr1*, *pfcr1*, *pfdhfr* and *pfdhps* alleles and treatment outcome**

To maximize our sample size, we pooled the data from both study sites and evaluated the association between infections with single and combined mutant alleles in *pfmdr1*, *pfcr1*, *pfdhfr* and *pfdhps* and response to treatment. All patient isolates were coded according to presence or absence of mutant alleles and isolates showing both, wild type and mutant allele, were treated as mutant. Likewise, infecting genotypes were coded according to the most highly mutated *pfmdr1*, *pfcr1*, *pfdhfr* and *pfdhps* alleles present in the sample.

Apart from *pfmdr1* N86Y (OR=9.26, 95% CI: 1.22-70.08,  $p<0.01$ ) and *pfdhps* A437G (OR=3.82, 95% CI: 1.62-9.01,  $p<0.01$ ), there was no independent marker found to be significantly associated with treatment failure (Table 3). When we adjusted for known confounding factors, such as initial parasite density and age, in a stepwise logistical regression model, the significant association for the two above-mentioned markers was retained (data not shown).

In a further step, we established the genetic profile of parasites for each patient sample. With regard to mutated gene loci in all four genes analysed, we could discriminate between 24 different genotypes (Table 4). Among those, eight were observed in treatment failure cases, whereas the remaining 16 were exclusively found in patients with an adequate treatment response. The investigation of the relationship between these genotypes and treatment failure revealed the following. Considering the genotypes with a fully wild-type *pfcr1*, the risk of treatment failure was slightly increased when concurrent mutations in *pfmdr1* N86Y and *pfdhfr* S108N+C59R were seen (OR=1.45, 95% CI: 0.27-7.74,  $p=0.67$ ). When the latter three

mutations were observed in conjunction with a triple (K76T+N326D+I356L) or quadruple (plus A220S) mutation in *pfcr*, the risk of treatment failure was not significantly changed (OR=0.83, 95% CI: 0.30-2.35,  $p=0.73$  and OR=1.18, 95% CI: 0.61-2.30,  $p=0.62$  for the *pfcr* triple and quadruple mutant, respectively). In contrast, the risk of failure with a triple or quadruple mutant in *pfcr* was significantly increased with the additional mutation A437G in *pfdhps* (OR=3.76, 95% CI: 0.73-19.32,  $p=0.12$  and OR=4.22, 95% CI: 1.56-11.39,  $p<0.01$ , respectively). In contrast, when the *pfcr* quadruple mutant was combined with the double mutation *pfdhfr* S108N+C59R, but showed wild-type alleles in *pfmdr1* N86Y and *pfdhps* A437G, the odds ratio for failure was decreased (OR=0.22, 95% CI: 0.03-1.72,  $p=0.08$ ). Furthermore, treatment failures were observed among *pfcr* quadruple plus *pfmdr1* N86Y mutants without any concurrent mutations in *pfdhfr* or *pfdhps* (OR=3.04, 95% CI: 0.78-11.85,  $p=0.12$ ).

To investigate whether the difference in treatment outcome at the two study sites was reflected in the drug resistance profile of the corresponding parasites, we calculated the mutant allele frequencies for each gene locus. Maximum likelihood estimates of mutant allele frequencies found in the two study populations are presented in figure 2. Regarding the allele frequencies for the CQ relevant molecular markers, there was no significant difference in *pfcr* K76T, N326D, and I356L. The only statistically significant differences in allele frequencies between the Karimui and the Wosera area were found for *pfcr* A220S (0.57 versus 0.81) and *pfmdr1* N86Y (0.99 versus 0.71). A similar picture was observed for the SP relevant molecular markers. Whereas the difference in any of the mutated loci in *pfdhfr* was not significant, the genetic profile for *pfdhps* mutation A437G was significantly different in the two parasite populations with an allele frequency of 0.26 in the Karimui area versus 0.02 in the Wosera area.

## DISCUSSION

This study investigated the relationship between polymorphisms in four malaria resistance related genes in *P. falciparum* and the *in vivo* response to treatment with the combination regimen of AQ or CQ plus SP. First, therapeutic efficacy was assessed in two rural areas in PNG which are different with regard to transmission intensity and drug use patterns, whereby we measured treatment failure rates of 28% in the Karimui and 16% in the Wosera area (Marfurt *et al.*, 2006, submitted, Chapter 2). The genetic drug resistance profile including 24 molecular markers was established in pre-treatment samples from both sites by the use of a new DNA microarray-based technology (Cramer *et al.*, 2006, submitted, Chapter 3) and its relationship with *in vivo* drug response was analysed in the pooled sample set. The principal objectives were to establish the baseline prevalence of polymorphisms in genes related to CQ and SP resistance, to assess their relationship with treatment outcome with combination therapy, in order to identify and propose useful markers for molecular monitoring of drug resistant *P. falciparum* in the country.

The analysis of the genetic profile of the parasite population revealed high levels of mutant alleles in CQ resistance (CQR) related *pfprt* and *pfmdr1* genes. Polymorphism in *pfmdr1* N86Y and *pfprt* K76T, the most important key markers for CQR, were found in 86% and 91% of all patients, respectively. Additional polymorphisms known to be associated with CQR, such as *pfprt* A220S, N326D and I356L, were found in 70%, 89% and 89% of all isolates. The long history of 4-aminoquinoline use as monotherapy in PNG, which was accompanied by accumulating reports about increasing levels of *in vivo* CQR (Müller *et al.*, 2003; Genton *et al.*, 2006), has led to a highly CQ resistant genetic background in the parasites as reported previously (Chen *et al.*, 2001; Mehlotra *et al.*, 2001; Mehlotra *et al.*, 2005; Nagesha *et al.*, 2003). Similarly high frequencies of mutated alleles in *pfmdr1* N86Y and *pfprt* K76T were reported recently in a study conducted in PNG between May 2000 and October 2001 (Casey *et al.*, 2004), where the analysis of parasites from treatment failure samples collected in town clinics in Maprik and Madang had shown mutation rates above 80% for these two CQR loci. In addition, our results demonstrated prevalence rates of 91% and 82% for mutant alleles in the pyrimethamine related gene loci *pfdhfr* S108N and C59R, which were higher than those reported in the afore mentioned study for Maprik (72% and 51%, respectively) and Madang (82% and 74%, respectively). More recently, Mita *et al.* (2006a) analysed *P. falciparum* isolates from patients attending two town clinics in Wewak, the provincial capital of the East

Sepik Province in PNG, and also observed rather high prevalence rates of *pf dhfr* double S108N+C59R mutations (83% in 2002 and 86% in 2003). These high levels of mutation rates in *pf dhfr* appearing only a short time after the implementation of SP as one component of the official first-line policy were not surprising. Prior drug exposure to antifolates has been thought to be low because SP was only used in combination with quinine as second line therapy against treatment failure and severe malaria. However, former drug pressure may have been exerted by the use of pyrimethamine (in combination with CQ) in mass drug administration campaigns in the 1960s and 1970s (Spencer, 1992). Moreover, cotrimoxazole, a combination regimen of trimethoprim-sulphamethoxazole, is a commonly used antibiotic to treat bacterial infections at health facilities in PNG. Since cross-resistance between pyrimethamine and trimethoprim has been described *in vitro* and on molecular level (Basco & Le Bras, 1997; Iyer *et al.*, 2001; Khalil *et al.*, 2003), widespread use of cotrimoxazole may have exerted additional selective pressure on *pf dhfr* in former years. Recent microsatellite analysis in *dhfr*-flanking regions by Mita *et al.* (2006a) revealed that the most prevalent *dhfr* haplotype (i.e., S108N+C59R double mutation) was associated with reduced microsatellite variability around the gene, an observation which argues for the selection of pre-existing SP resistant parasites, rather than the frequent emergence of *de novo* mutations in this gene (Hastings *et al.*, 2002; Pearce *et al.*, 2005). These data further corroborate the hypothesis, that former drug pressure has lead to the emergence of pyrimethamine resistant parasites before the official introduction of SP in PNG.

In addition to high prevalence rates of genetic loci reflecting high CQR and moderately reduced pyrimethamine sensitivity, we also describe the occurrence of polymorphic alleles in the sulphadoxine resistance related gene *pf dhps*. Until 2003, polymorphic *pf dhps* loci associated with reduced sensitivity to sulpha drugs have only been found in a single *P. falciparum* isolate originating from PNG (Casey *et al.*, 2004; Reeder *et al.*, 1996). In our pre-treatment samples collected between 2003 and 2004, we measured prevalence rates of 13% for A437G and 1% for K540E. Likewise, Mita and colleagues detected mutations in these loci in 8% of patient isolates collected in Wewak in the year 2003 (Mita *et al.*, 2006a). In contrast to our results, they found a strong linkage between the two mutated *dhps* alleles and always detected them in conjunction with the double mutation S108N+C59R in *pf dhfr*. Though the A437G mutation in *pf dhps* is thought to be the first selected mutation under sulphadoxine pressure (Kyabayinze *et al.*, 2003; Nzila *et al.*, 2000; Triglia *et al.*, 1997), two samples in our study harboured *dhps* genotypes having a single K540E mutation without a concomitant A437G mutation. Though very uncommon, the same genotype has been described previously

in patient isolates from Tanzania, Malawi, and Sudan (Bwijo *et al.*, 2003; Dorsey *et al.*, 2004; Wang *et al.*, 1997). In the view that *pfdhfr* mutations usually predominate over those in *pfdhps* (Wang *et al.*, 1997), the detection of genotypes having a single *dhps* A437G mutation in combination with a *pfdhfr* wild type allele in two of our samples was rather unusual. However, this genotype may well have been selected by sulpha drugs used to treat infectious diseases other than malaria.

Most data concerning the relationship between molecular markers and treatment outcome have been produced by the genetic analysis of patient isolates originating from clinical trials evaluating the efficacy of a single drug class. Straightforward associations have been rarely found and therefore, the elucidation of an association between molecular correlates and treatment outcome with combination regimens containing drug classes being effective against different parasite targets may be even more complex. In order to propose a suitable marker set for the molecular monitoring of *P. falciparum* against the current first-line combination therapy with AQ or CQ plus SP, we investigated the association of single mutations as well as infecting genotypes (i.e., combinations of mutated alleles in the respective genes) with *in vivo* treatment response. Regarding CQ relevant markers, the only single marker associated with a significantly increased risk of treatment failure was *pfmdr1* N86Y. Taking into account additional SNPs in *pfcr1*, neither of the mutated alleles increased the predictive value for *pfmdr1* N86Y, the most likely reason being that these mutations nearly reached fixed levels in the parasite population. Similarly, pyrimethamine relevant markers in *pfdhfr* did not show a significant association with treatment failure. Risk of failure was only increased with infections harbouring the A437G mutation in *dhps*. These observations are in agreement with previous studies showing that the prevalence of single molecular markers (e.g. *pfcr1* K76T or *pfdhfr* S108N) was almost always higher than the level of clinical or parasitological resistance to the respective drugs, especially in regions with high transmission intensity and long lasting drug pressure (Djimde *et al.*, 2001; Mayor *et al.*, 2001; Rallon *et al.*, 1999) and therefore, renders these markers unsuitable for molecular monitoring. Furthermore, the validity of molecular markers is dependent on former drug use and may also vary according to the malaria epidemiology in a given area (Alifrangis *et al.*, 2003; Omar *et al.*, 2001; Staedke *et al.*, 2004). The relevance of these epidemiological characteristics is further illustrated by the following conflicting results. Similar to our data, a significant association between *pfdhps* A437G and treatment response with CQ plus SP was found in a study conducted in Laos (OR=15.00, 95% CI=1.23-412.69) (Berens *et al.*, 2003). In contrast, the K540E mutation in *pfdhps* was shown to be a better indicator of treatment failure with the same combination

regimen in Uganda (Dorsey *et al.*, 2004). The evaluation and assessment of a combination of markers (e.g. quintuple mutation *pf dhfr* S108N+C59R+N51I plus *pf dhps* A437G+K540E, or *pf crt* K76T in combination with *pf mdr1* N86Y), instead of single markers indicating the presence of a highly resistant genotype, have been suggested for the molecular monitoring of antimalarial resistance (Jelinek *et al.*, 2002; Khalil *et al.*, 2005; Kublin *et al.*, 2002, Kyabayinze *et al.*, 2003; Nzila *et al.*, 2000; Talisuna *et al.*, 2004). In our study, which took into account the combined *pf crt/pf mdr1/pf dhfr/pf dhps* genotype, the risk of treatment failure was clearly associated with the total number of mutations in the analysed genes. But the risk was only significantly increased for patients who harboured parasites with the most highly mutated genotype (i.e., 8/24 SNPs mutated).

Unusual findings in our study included the observation of treatment failures with genotypes having either a fully wild type *pf crt* combined with a mutated *pf mdr1* N86Y plus the double *pf dhfr* S108N+C59R mutation, or a fully wild type *pf dhfr*+*pf dhps* allele combined with the N86Y mutation in *pf mdr1* and the quadruple mutation in *pf crt*. These results highlight again the fact that among many parasite and host factors, the molecular resistance background of *P. falciparum* is only one of several determinants for *in vivo* treatment outcome. Whereas acquired immunity can account for the clearance of drug resistant genotypes, diminished drug metabolism may well explain treatment failure in spite of an infection with a susceptible genotype (Cravo *et al.*, 2001; Djimde *et al.*, 2003; White, 2002).

Regarding former drug history in PNG (i.e., long lasting 4-aminoquinoline use and sporadic use of SP) which has led to a highly CQ and moderately SP resistant genetic background in the parasite population, the relevance of key *pf dhps* mutations in predicting treatment failure was expected. AQ and CQ as inefficacious partner drugs of SP in the new standard regimen were not able to curb both, the progression of pyrimethamine resistance as well as the emergence of sulphadoxine resistance. It is most likely that in our *in vivo* studies, we measured the clinical efficacy of the sulpha component. Therefore, with ongoing drug pressure with the current first-line policy, the assessment of SNPs related to sulphadoxine resistance will be an important molecular index for increasing resistance in PNG. However, according to our results, also *pf mdr1* N86Y plays an important role in predicting a negative treatment response. Linkage disequilibrium between the key CQR markers *pf crt* K76T and *pf mdr1* N86Y has been reported by several authors (Babiker *et al.*, 2001; Djimde *et al.*, 2001; Fidock *et al.*, 2000; Happi *et al.*, 2006a; Mita *et al.*, 2006b) and has led to the conclusion that the latter mutation might either compensate for fitness costs induced by the K76T mutation in *pf crt* or augment CQR in the parasite (Cooper *et al.*, 2005; Walliker *et al.*, 2005). CQ and AQ

are chemically related drugs and cross-resistance has been described in several clinical and *in vitro* reports (Basco & Le Bras, 1993; Olliaro *et al.*, 2003). Though little is known about the genetic mechanisms conferring AQ resistance (Meshnick & Alker, 2005), an important role has been ascribed to the key CQR markers *pfprt* K76T and *pfmdr1* N86Y (Dokomajilar *et al.*, 2006; Ochong *et al.*, 2003). It has been shown recently that in combination with *pfprt* K76T, the *pfmdr1* N86Y polymorphism was predictive for treatment failure with AQ in Nigeria (Happi *et al.*, 2006b) and that AQ resistance was associated with the selection of these polymorphisms in Kenya (Holmgren *et al.*, 2006). Considering the long use of AQ as monotherapy against uncomplicated falciparum malaria in PNG and our observation that *pfmdr1* N86Y is a strong predictor for treatment failure with AQ plus SP, our data support the hypothesis that *pfmdr1* N86Y is probably involved in AQ resistance. Several studies have shown that both, SNPs and gene amplification of *pfmdr1*, can mediate resistance to 4-aminoquinolines and also other drug classes, such as amino alcohols and artemisinin derivatives (Duraisingh *et al.*, 2000; Foote *et al.*, 1989; Foote *et al.*, 1990; Price *et al.*, 2004; Reed *et al.*, 2000). However, results from different studies investigating the relationship of these genetic alterations in *pfmdr1* and *in vivo* response were often inconsistent (Flück *et al.*, 2000; Happi *et al.*, 2003; Pillai *et al.*, 2001; Tinto *et al.*, 2003). Several possible direct (active drug translocation) or indirect (modification of biophysical cell parameters) modes of action have been proposed for P-glycoprotein homolog 1, the gene product of *pfmdr1*. But how genetic alterations in *pfmdr1* and epistatic interactions with other genes finally lead to a multidrug resistant phenotype, this question still remains to be resolved (Duraisingh & Cowman, 2005; Duraisingh & Refour, 2005; Roepe, 2000).

Finally, we were interested to see whether the observed difference in clinical outcome between sites was reflected in the genetic profile of the corresponding parasite populations. By taking into account the varying multiplicity of infection, we estimated and compared allele frequencies for all the mutated gene loci measured at both sites. Thereby we could show that the levels of treatment failure rate were reflected by statistically significant differences in frequencies of *pfmdr1* N86Y and *pfdhps* A437G. These data further confirmed the role of these two markers as important predictors for a negative treatment response with AQ or CQ plus SP and suggests them to be the most useful resistance surveillance markers with the current standard treatment in PNG.

To recapitulate, with regard to the drug use history in PNG, we describe a genetic background in the parasite population that is associated with high CQ as well as moderate pyrimethamine resistance. Moreover, the emergence of mutations concordant with a sulphadoxine resistant

phenotype indicates that the efficacy of the sulpha component is already compromised. Therefore, under sustained drug pressure with both drug classes, close *in vivo* as well as molecular monitoring with the suggested markers is highly recommended so that appropriate measures can be taken in due time. We have shown that a careful baseline assessment of molecular markers including the investigation of their relationship with treatment response is important for the identification of appropriate marker sets. For that purpose, DNA microarray technology has been proven to be a valuable and cost-effective tool for the parallel analysis of SNPs in multiple genes in a large sample size. However, the use of additional markers could become necessary for the longitudinal resistance monitoring in the future, in particular when current drug policy will be changed. These may include SNPs in known or as yet uncharacterized genes involved in resistance to the commonly used antimalarials (Mu *et al.*, 2003), or markers against newly implemented drug classes, such as the artemisinins (Jambou *et al.*, 2005; Uhlemann *et al.*, 2005).



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**TABLE 1:** Baseline characteristics of study sites and patients at enrolment

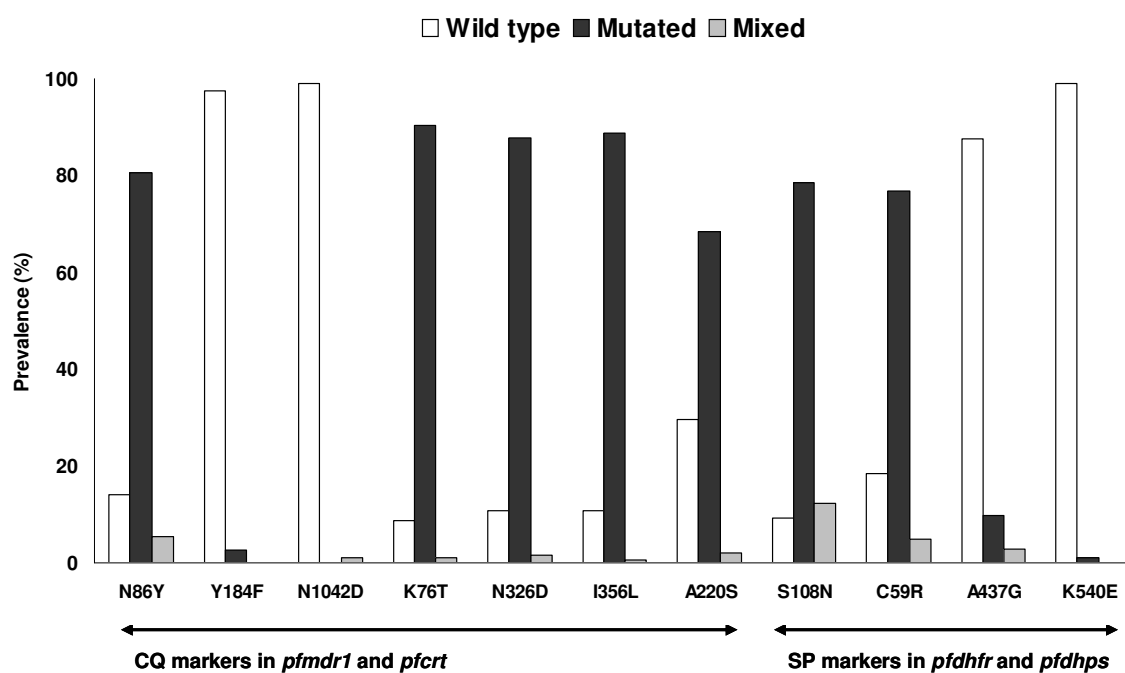
<b>Characteristics</b>	<b>Study site</b>	
	<b>Karimui area</b> (Simbu Province) n=97	<b>South Wosera</b> (East Sepik Province) n=112
<b><i>Study sites</i></b>		
Endemicity*	mesoendemic	mesoendemic
Transmission intensity <sup>§</sup>	moderate	high
<b><i>Patients</i></b>		
Weight (mean (95% CI), kg)	13.8 (12.9-14.6)	14.4 (13.8-15.1)
Age (mean (95% CI), yrs)	4.0 (3.7-4.4)	4.5 (4.2-4.8)
Sex: female/n (%)	43/97 (44.3)	59/112 (52.7)
Temperature (mean (95% CI), °C)	38.7 (38.5-38.9)	38.7 (38.4-39.0)
Hb ( mean (95% CI), g/dl)	9.0 (8.6-9.5)	9.0 (8.7-9.3)
Parasite density (geometric mean (range), per µl)	21937 (1120-329400)	40526 (280-774400)
Multiplicity of infection (=MOI) (mean (95% CI))	1.48 (1.34-1.63)	1.73 (1.59-1.88)
Spleen rate <sup>#</sup> (%) (95% CI)	43.3 (33.3-53.7)	50.9 (41.3-60.5)

\* Assessed by concomitant cross-sectional surveys in both study areas which showed *P. falciparum* prevalence rates of 11-50% in children aged 2-9 years (WHO, 2003); <sup>§</sup> Müller *et al.*, 2003; <sup>#</sup> proportion of children with enlarged spleen

**TABLE 2:** Treatment outcomes for amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against *P. falciparum* malaria in Papua New Guinea

	Study sites	
	Karimui area	South Wosera area
	n=97	n=112
<b>Treatment</b>	x/n (%)	
AQ plus SP	80 (82.5)	94 (83.9)
CQ plus SP	17 (17.5)	18 (16.1)
<b>Outcome*</b>	x/n (%)	
ACPR <sup>a</sup>	70 (72.2)	94 (83.9)
ETF <sup>b</sup>	2(2.1)	5 (4.4)
LCF <sup>c</sup>	7 (7.2)	1 (0.9)
LPF <sup>d</sup>	18 (18.5)	12 (10.8)
Total TF <sup>e</sup>	27 ( <b>27.8</b> )	18 ( <b>16.1</b> )
New infections	3 (10.7)	4 (23.5)

AQ, amodiaquine; SP, sulphadoxine-pyrimethamine; CQ, chloroquine; \* PCR-corrected values up to Day 28; <sup>a</sup> ACPR, Adequate clinical and parasitological response; <sup>b</sup> ETF, Early treatment failure; <sup>c</sup> LCF, Late clinical failure; <sup>d</sup> LPF, Late parasitological failure; <sup>e</sup> TF, Treatment failure



**FIGURE 1:** Prevalence of mutations in *pfmdr1*, *pfcr1*, *pfdhfr* and *pfdhps* in patient samples from Papua New Guinea. CQ, chloroquine; SP, sulphadoxine-pyrimethamine; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1; *pfcr1*, *Plasmodium falciparum* chloroquine resistance transporter; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase; no mutation was detected in any of the other SNP sites analysed (13/24 sites)

**TABLE 3:** Association between mutated single markers in *pfcr*, *pfmdr1*, *pfdhfr* and *pfdhps* and treatment outcome with amodiaquine or chloroquine plus sulphadoxine-pyrimethamine

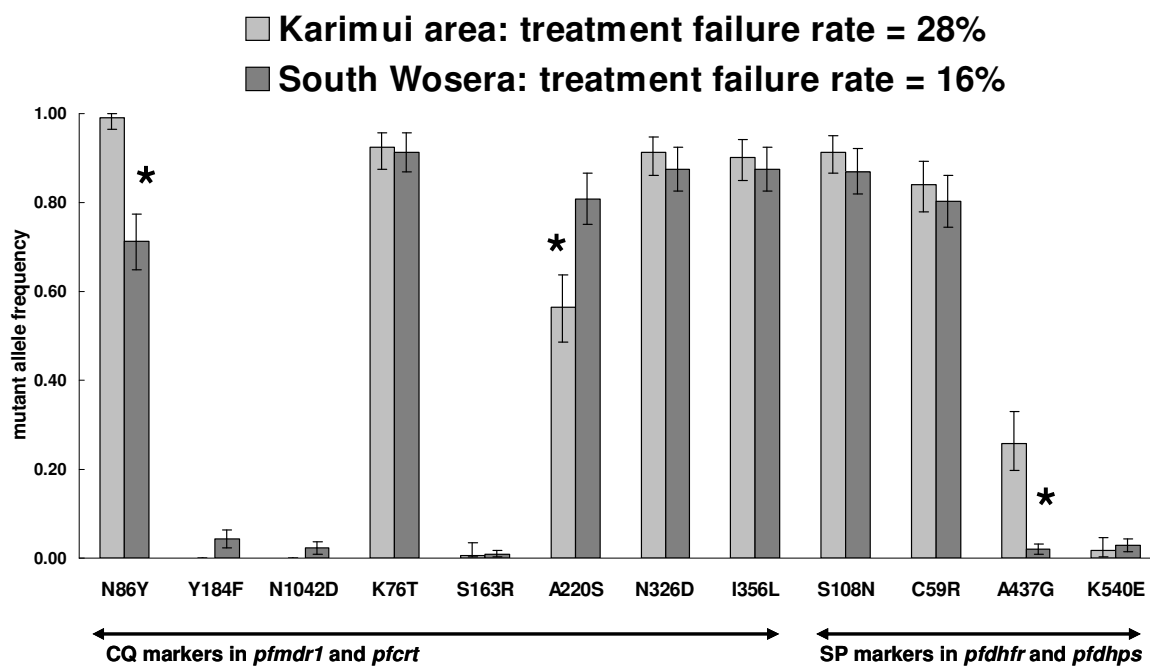
Gene	polymorphism	OR	95 % Confidence Interval	<i>p</i> (LRT)
<i>pfcr</i>	K76T	2.37	0.52-10.73	0.22
<i>pfcr</i>	I356L	3.05	0.69-13.57	0.10
<i>pfcr</i>	N326D	3.05	0.69-13.57	0.10
<i>pfcr</i>	A220S	1.62	0.75-3.53	0.22
<i>pfmdr1</i>	N86Y	9.26	1.22-70.08	<b>&lt;0.01</b>
<i>pfmdr1</i>	Y184F	§		
<i>pfmdr</i>	N1042D	§		
<i>pfdhfr</i>	S108N	1.05	0.33-3.35	0.93
<i>pfdhfr</i>	C59R	2.06	0.75-5.64	0.13
<i>pfdhps</i>	A437G	3.82	1.62-9.01	<b>&lt;0.01</b>
<i>pfdhps</i>	K540E	§		

OR, odds ratio; LRT, likelihood ratio test; *pfcr*, *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase; § mutated alleles were not detected in samples from treatment failure cases

**TABLE 4:** Association between infecting *pfprt*, *pfmdr1*, *pfdhfr* and *pfdhps* genotypes and treatment outcome with amodiaquine or chloroquine plus sulphadoxine-pyrimethamine

CQ-relevant markers					SP-relevant markers							
<i>pfprt</i>				<i>pfmdr1</i> *	<i>pfdhfr</i>		<i>pfdhps</i>					
K76T	N326D	I356L	A220S	N86Y	S108N	C59R	A437G	K540E	P (%)	OR	95%CI	<i>p</i> ( $\chi^2$ )
									1.46	§		
									0.49	§		
									1.94	§		
									0.49	§		
									0.49	§		
									0.49	§		
									0.49	§		
									0.49	§		
									0.49	§		
									7.77	0.22	0.03-1.72	0.08
									0.49	§		
									0.97	§		
									3.40	1.45	0.27-7.74	0.67
									0.97	§		
									0.97	§		
									1.46	§		
									12.62	0.83	0.30-2.35	0.73
									2.91	3.76	0.73-19.32	0.12
									0.49	§		
									4.37	3.04	0.78-11.85	0.12
									0.97	§		
									5.83	0.31	0.04-2.47	0.20
									41.26	1.18	0.61-2.30	0.62
									8.74	4.22	1.56-11.39	<0.01
									0.49	§		

\* due to very low mutation rates, genotypes with mutated gene loci Y184F and N1042D in *pfmdr1* were grouped together with the wild type *pfmdr1* genotypes; CQ, chloroquine; AQ, amodiaquine; SP, sulphadoxine-pyrimethamine; *pfprt*, *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase; P, prevalence; OR, odds ratio; white box, wild-type allele; grey box, mutated allele; § the genotype was not detected in samples from treatment failure cases



**FIGURE 2:** Maximum likelihood estimates of mutant allele frequencies at the two study sites. Error bars denote 95% confidence intervals; \* denotes statistical significance at the 95% level; CQ, chloroquine; SP, sulphadoxine-pyrimethamine; *pfmdr1*, *Plasmodium falciparum* multidrug resistance gene 1; *pfcr1*, *Plasmodium falciparum* chloroquine resistance transporter; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase

## Chapter 5

### **Community versus clinical molecular monitoring of parasite resistance to antimalarial drugs**

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## SUMMARY

**OBJECTIVE** To test the hypothesis that parasite populations circulating in the community have the same genetic profile for resistance markers as the ones collected from malaria patients attending health facilities.

**METHODS** Assessment of twenty-four molecular markers known to be associated with resistance to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) was done by using a novel DNA microarray-based technology. Mutant allele frequencies were estimated by a Markov Chain Monte Carlo algorithm and genetic profiles of parasite populations circulating in clinical and community samples from two sites in Papua New Guinea were compared.

**RESULTS** Different treatment failure rates with the current first-line regimen of CQ plus SP observed at the two study sites were reflected in the genetic profile of the corresponding parasites in clinical samples. There was no difference in the genetic resistance background of parasites collected from clinical or community samples.

**CONCLUSION** Molecular monitoring of drug resistant malaria can be accomplished much quicker and easier by the use of a community approach and a technology to measure a wide array of drug resistance markers in large sample sets. The implementation of a community approach enables surveillance to be expanded to remote areas with limited access to health care facilities, and thus can contribute to a more comprehensive assessment of parasite resistance to antimalarial drugs countrywide.



## INTRODUCTION

The burden of malaria has been increasing in recent years, the main reason being the development and spread of *P. falciparum* resistance to the most commonly used antimalarial drugs, such as the 4-aminoquinolines and the antifolates (Olliaro, 2005; Trape, 2001). Knowledge about the level of parasite resistance to drugs through regular surveillance is necessary to make decisions on drug policies and treatment strategies. Methods for the assessment of parasite resistance in malaria include *in vivo* drug efficacy studies, which are still the gold standard, and to a lesser extent, *in vitro* sensitivity testing of patient isolates. Since parasite resistance is frequently associated with single nucleotide polymorphisms (SNPs) within coding genes for important drug targets or transporter proteins, the assessment of molecular markers has become an important complementary tool for the monitoring of drug resistance (Plowe, 2003; Plowe, 2005; WHO, 2005).

Apart from the known limitations of *in vivo* drug efficacy studies, such as the assessment of one drug regimen only, and the fact that they are resource- and time-consuming, analysis is restricted to a biased sample with regard to study population and area (Fevre & Barnish, 1999). Considering the study population, analysis is restricted to clinical cases and few age categories (i.e., children < 5 years). Parasites circulating in asymptomatic carriers are therefore neglected with a health facility-based approach. Moreover, clinical studies are usually conducted at selected sentinel sites with good access to health care facilities, and remote areas are thus excluded from surveillance. A community-based monitoring approach could give a more comprehensive assessment of the genetic profile of the parasite population circulating in a given area and contribute to a better understanding of the relationship between drug resistance genotypes and level of clinical failure rates as well as the dynamics and spread of drug resistant malaria.

Most molecular analyses have been done in pre-treatment samples collected from clinical studies and were limited in the number of molecular markers studied. For the analysis of molecular markers in large scale epidemiological studies, such as community surveys conducted in several areas, there is a need for new, easy to use and cheap high throughput methods which allow the parallel analysis of several SNPs in large sample sets.

Molecular studies assessing antimalarial drug resistance markers up to date have been done with clinical samples and there is as yet no indication whether the molecular profile of parasites circulating in the community matches the profile of those observed among patients

attending health facilities. In order to evaluate the feasibility of a community-based monitoring approach, we tested the hypothesis whether parasite genotypes circulating in the whole community were similar to those circulating in clinical malaria cases. For this purpose, we first established the molecular drug resistance profile in community and clinical samples by using a newly developed DNA microarray-based technology and then compared the genetic profiles of the two parasite populations in two sites in Papua New Guinea, which had shown different *in vivo* treatment failure rates with the current first-line treatment of amodiaquine (AQ) or CQ plus SP.

## PATIENTS, MATERIALS, AND METHODS

Studies were done at two sites in Papua New Guinea between October 2003 and April 2004. Both selected sites, the Karimui area in the Simbu Province and the South Wosera area in the East Sepik Province, are rural places mesoendemic for malaria but differ with regard to transmission intensity and drug use patterns (Genton *et al.*, 1995; Mehlotra *et al.*, 2002; Müller *et al.*, 2004).

Clinical samples were collected within drug efficacy studies conducted according to the standardised World Health Organization (WHO) protocol for low to moderate transmission areas (WHO, 2003), as described in detail elsewhere (Marfurt *et al.*, 2006, submitted; Chapter 2). Community samples were collected from cross-sectional surveys conducted in the catchment areas of the health facilities at both sites using a randomized household approach. To obtain a representative sample of the parasite population circulating in the corresponding communities (i.e., approximately 100 PCR-positive *P. falciparum* samples from each location), we collected between 300 and 350 blood specimens per community (Mehlotra *et al.*, 2002).

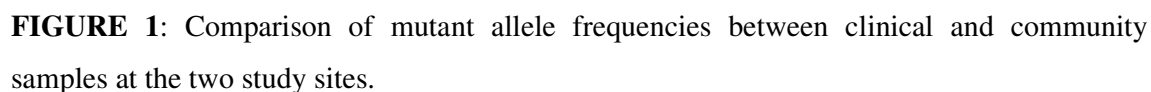
Assessment of SNPs for drug resistant malaria was done for *pfmdr1* codons N86Y, Y184F, S1034C, N1042D and D1246Y, *pfprt* codons K76T, H97Q, T152A, S163R, A220S, Q271E, N326D/S, I356L/T and R371I, *pfdhfr* condons A16V, N51I, C59R, S108N/T and I164L, and *pfdhps* codons S436A, A437G, K540E, A581G, and A613T/S. The method was based on parallel PCR amplification of the target sequences followed by primer extension mediated mini-sequencing using fluorochrome-labelled ddNTPs. Subsequent base calling occurred on a microarray upon sequence specific hybridisation (Crameri *et al.*, 2006, submitted, Chapter 3; Marfurt *et al.*, 2006, in preparation, Chapter 4).

Statistical analyses were performed by the use of STATA software (version 8.2; Stata Corp., College Station, Texas). To allow for the varying multiplicity of infection, which was determined by genotyping of the highly polymorphic *msp2* locus (Felger & Beck, 2002; Cattamanchi *et al.*, 2003), a Markov Chain Monte Carlo algorithm was used to obtain estimates of mutant allele frequencies ( $p$ ). Differences of allele frequencies between parasite populations in clinical and community samples were assessed by the comparison of Bayesian confidence intervals (CI) for  $p$  (Schneider *et al.*, 2002; Marfurt *et al.*, 2006, in preparation, Chapter 4).

## RESULTS

PCR-corrected treatment failure rates up to day 28 with AQ or CQ plus SP for *P. falciparum* malaria were 28% in the Karimui and 16% in the Wosera area (Marfurt *et al.*, 2006, submitted, Chapter 2). We had previously identified the strongest independent predictors for treatment failure with AQ or CQ plus SP to be *pfmdr1* N86Y and *pfdhps* A437G. Moreover, we could demonstrate that the difference in clinical outcome was reflected in the corresponding molecular drug resistance profile of the parasite populations derived from clinical samples. Significant differences between the Karimui and the Wosera area were not only seen in the mutant allele frequencies of CQ resistance markers *pfcr1* A220S (0.57 versus 0.81) and *pfmdr1* N86Y (0.99 versus 0.71). A similar picture was observed for the SP relevant marker *pfdhps* A437G with an allele frequency of 0.26 in the Karimui area versus 0.02 in the Wosera area (Marfurt *et al.*, 2006, in preparation, Chapter 4).

In order to test the hypothesis that there is no difference between the genetic drug resistance profile in community and clinical samples, we simultaneously determined the profile of molecular markers in the respective community samples. We then compared the estimated mutant allele frequencies for each SNP in clinical and community samples. Thereby we showed that there was no difference in mutant allele frequency for any of the SNPs analysed between community and clinical samples. We also demonstrated the match of these two genetic profiles for both study sites which differ with regard to transmission intensity and drug use patterns (Figure 2).



n, number of samples analysed; A, Karimui area; B, South Wosera area; Error bars denote Bayesian confidence intervals (95%) for mutant allele frequencies; CQ, chloroquine; SP, sulphadoxine-pyrimethamine; *pfmdr1*, *Plasmodium falciparum* multi drug resistance gene 1; *pfcr*, *Plasmodium falciparum* chloroquine resistance transporter gene; *pfdhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pfdhps*, *Plasmodium falciparum* dihydropteroate synthase

## DISCUSSION

On an individual level, an association of specific molecular markers with *in vitro* resistance does not allow unambiguous prediction of *in vivo* therapeutic response, since a number of other parameters, such as drug use patterns, level of prior immunity, intensity of transmission, or compliance to treatment, play a role in clearing symptoms and parasites (Djimde *et al.*, 2003; Hastings & Watkins, 2005; White, 2004). Furthermore, most current models postulate the molecular basis of antimalarial resistance to be multigenic (Duraisingh & Refour, 2005; Mu *et al.*, 2003). However, for regular surveillance of drug resistant malaria, the monitoring of molecular markers is considered a valuable complementary tool to the classical methods, such as *in vivo* drug efficacy studies and *in vitro* sensitivity testing (WHO, 2003; WHO 2005). The majority of previous studies investigating molecular correlates of antimalarial resistance have been done with samples collected in clinical studies. As a consequence, analysis was frequently restricted to a sub-sample of the whole population (i.e., symptomatic cases in the age group below 5 years). Therefore, a large parasite reservoir (i.e., parasites circulating in the untreated asymptomatic population), which nonetheless plays an important role in the development and spread of resistance, was not investigated. Furthermore, most of these clinical-based studies were conducted in a relatively small number of sentinel sites and thus neglected remote areas with limited access to health care facilities. More recently, the comparability of clinical and community molecular data was further challenged by Bwijo and colleagues (2003) who put forward the following concept. Because symptomatic patients attending health care facilities are more prone to have taken antimalarial drugs before and are thus more likely to harbour resistant parasites selected by drug treatment, resistant genotypes might be over-represented in clinical samples. Nevertheless, a community-based approach for molecular surveillance has been suggested by several authors and based on correlations found between population-based molecular data and the level of clinical failure, molecular surveillance indices have been proposed (Djimde *et al.*, 2001; Talisuna *et al.*, 2002; Talisuna *et al.*, 2003; Pearce *et al.*, 2003). But due to technical limitations, these studies have only assessed a small number of gene loci and were therefore considerably limited in drawing conclusions for further drug policy recommendations.

In order to evaluate the validity of a broader community-based surveillance approach, it was for us an important prerequisite to show that the molecular drug resistance profile assessed in clinical samples does not differ from that in community samples. To cover multiple resistance

markers in different genes, we applied a newly developed DNA microarray-based technology and simultaneously analysed 24 SNPs in four different genes in a total of 418 samples. We demonstrated in the current report that there was no difference in the estimates of mutant allele frequencies in parasite populations collected from symptomatic children between 6 months and 7 years of age or from a random sample of the whole community. In other words, the different levels of *in vivo* resistance, which had previously been shown to be reflected in the genetic resistance profile of the corresponding clinical samples, were also mirrored in the profile detected in community samples. Thus, molecular monitoring of parasite resistance is feasible by using cross-sectional surveys.

Such community-based cross-sectional surveys applying highly standardized protocols with regard to sample size, sentinel sites and genetic analysis can be done in a quick and cost-effective way by the application of our newly developed DNA microarray-based method and provides a new tool as early warning system for drug resistance. This technology does not only allow the simultaneous analysis of markers for several different drug classes, but is also highly flexible since markers can be easily added or deleted according to specific monitoring needs. Such population-based data collected over time could capture the dynamics of resistance, such as the emergence and/or disappearance of given genotypes to different drug classes (Bwijo *et al.*, 2003; Mita *et al.*, 2003; Laufer & Plowe, 2004) and could significantly add to the decision making process on when and where standard treatment should be changed and more importantly, on what drug classes an alternative treatment regimen should be based. Ongoing studies in different epidemiological settings in Papua New Guinea, the Solomon Islands and Tanzania are currently validating the usefulness and feasibility of our proposed community-based surveillance approach.

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## Chapter 6

### ***Plasmodium falciparum* resistance to antimalarial drugs in Papua New Guinea: Evaluation of a community-based approach using DNA microarray technology for the monitoring of resistance**

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## ABSTRACT

**BACKGROUND** Surveillance of drug resistance is an integral part of national malaria control programs in endemic countries and molecular monitoring of parasite resistance has become an important complementary tool in establishing rational drug policies. The principal aim of this study was to evaluate a community-based approach using a novel high throughput technology for the analysis of molecular markers in the parasite as a means for monitoring antimalarial resistance.

**METHODS** Between 2003 and 2005, we determined *in vivo* drug efficacy of amodiaquine (AQ) or chloroquine (CQ) plus sulphadoxine-pyrimethamine (SP) at three different sites in Papua New Guinea. We concurrently assessed the genetic drug resistance profile in community samples collected in the catchment areas of the respective health facilities by using a novel DNA microarray-based method for the parallel analysis of 33 single nucleotide polymorphisms (SNPs) in five different *P. falciparum* resistance associated genes (i.e., *pfcr*, *pfmdr1*, *pfdhfr*, *pfdhps*, and *pfATPase6*). We determined mutant allele frequencies and genotype patterns and investigated the relationship with corresponding treatment failure rate at each site in each year.

**RESULTS** PCR-corrected *in vivo* treatment failure rates with AQ or CQ plus SP were between 12% and 28%, depending on the respective site and year, and showed variable longitudinal trends at two sites. Clinical failure rates with the current combination regimen were reflected in the corresponding genetic resistance pattern of parasites from community samples. Frequencies of mutated alleles of markers in *pfcr* and *pfmdr1*, known to confer resistance to AQ/CQ, were high and did not show significant changes over time. Mutant allele frequencies in the pyrimethamine relevant gene *pfdhfr* were moderate at all three sites and those in *pfdhps*, involved in resistance to sulphadoxine, were still low, but showed different levels between sites. The opposing longitudinal trends in clinical response observed at two sites were best reflected by the frequencies and genotype patterns of mutations in SP relevant genes *pfdhfr* (S108N plus C59R) and *pfdhps* (A437G). Mutations in *pfATPase6*, the gene encoding a putative target for artemisinin derivatives, were not detected.

**CONCLUSION** In areas with high level of background AQ/CQ resistance, the evaluation of the frequency of 4-aminoquinoline relevant molecular markers in the community was not helpful to predict treatment response towards AQ or CQ plus SP, probably because polymorphisms in these genes had already reached fixed levels. In contrast, the estimation of mutation

frequencies in relevant SP resistance genes better mirrored treatment failure rates observed at nearby health facilities. Thus, indicators based on molecular data have to be considered with caution and interpreted in the local context, especially with regard to prior drug usage and level of pre-existing immunity. The community approach using relevant molecular markers for drugs that are likely to be still partially effective is a complementary tool that should help decision-making for the best treatment option and appropriate potential alternatives.

## INTRODUCTION

The burden of malaria has been increasing in recent years, the main reason being the development and spread of *P. falciparum* resistance to the most commonly used antimalarial drugs, such as the 4-aminoquinolines and the antifolates (Olliaro, 2005; Trape, 2001). Drug resistance is a major challenge in the control of malaria because effective drugs are the single most important tool to treat and control the disease in endemic countries. Rational drug policy is therefore a key issue in affected areas and knowledge about the level of parasite resistance to antimalarial drugs through regular monitoring is necessary to provide health authorities with reliable indicators of drug efficacy. Combination therapy as a means to improve clinical effectiveness and delay the emergence and spread of resistance to the individual drugs has been advocated for some years and successful implementation has been achieved in several countries (Kremsner & Krishna, 2004; White, 1999; WHO, 2006). Rapid, easy to use, and affordable surveillance systems are not only important to monitor emergence and spread of resistance to the newly adopted artemisinin-containing combination regimens (Adjuik *et al.*, 2004), but also decreasing resistance to withdrawn drugs, with the prospect of possibly reusing the limited number of safe and cheap drugs as partner compounds in antimalarial combination regimens (Laufer & Plowe, 2004). In addition, more comprehensive approaches are needed in order to assess the impact of new drug-based malaria intervention strategies such as intermittent preventive treatment (IPT) in infants and pregnant women (Schellenberg *et al.*, 2006).

Methods for the assessment of drug resistance in malaria include *in vivo* drug efficacy studies, which are still the gold standard, and to a lesser extent, *in vitro* sensitivity testing of patient isolates. More recently, the analysis of molecular markers has been proposed as an alternative approach for the evaluation of resistance to treatment (Plowe, 2003; Wernsdorfer & Noedl, 2003). Indeed, antimalarial resistance is often associated with point mutations in parasite genes encoding drug target molecules or transporters. Thus, the presence of specific point mutations in these genes can be used as indicators for treatment failure or decreased antimalarial efficacy.

Chloroquine resistance (CQR) is conferred by single nucleotide polymorphisms (SNPs) in *pfcr* (*Plasmodium falciparum* chloroquine resistance transporter) and *pfmdr1* (*P. falciparum* multidrug resistance 1), both encoding transport proteins localized in the digestive vacuole of the parasite (Fidock *et al.*, 2000; Foote *et al.*, 1990; Reed *et al.*, 2000; Sidhu *et al.*, 2002;

reviewed in Cooper *et al.*, 2005 and Duraisingh & Cowman, 2005). Resistance to sulphadoxine-pyrimethamine (SP) is associated with a stepwise accumulation of mutations in *pfdhfr* (*P. falciparum* dihydrofolate reductase) and *pfdhps* (dihydropteroate synthase), both encoding important enzymes of the parasite's folate synthesis pathway (Cowman, *et al.*, 1988; Peterson *et al.*, 1988; Triglia *et al.*, 1997; Triglia *et al.*, 1998; reviewed in Gregson & Plowe, 2005). Artemisinin derivatives have been shown to inhibit the sarcoendoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) of *P. falciparum* (*pfATPase6*) (Eckstein-Ludwig *et al.*, 2003). A finding which was further corroborated by more recent experiments demonstrating that decreased *in vitro* susceptibility to artemisinin derivatives was associated with SNPs in *pfATPase6* (Jambou *et al.*, 2005; Uhlemann *et al.*, 2005).

However, the usefulness of these molecular markers has been controversial because straightforward associations with *in vivo* treatment outcome were not consistently found in different epidemiological settings (Aubouy *et al.*, 2003; Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003; Pillai *et al.*, 2001; Tinto *et al.*, 2003; Wellems & Plowe, 2001). Reports may be conflicting because clinical outcome is also dependent on several host factors, such as pre-existing immunity, pharmacogenetic background determining drug metabolism, and compliance to treatment (White, 2004), as well as transmission intensity and drug use history in a given area (Alifrangis *et al.*, 2003; Pearce *et al.*, 2003). Furthermore, most previous studies focusing on molecular markers as indicators of treatment failure were conducted within the framework of clinical trials, and were therefore not only limited to sentinel sites with good access to health care facilities, but also investigated predominantly clinical cases within a restricted age group (i.e., children between 0.5 and 5 years of age) (Fèvre & Barnish, 1999). Hence, a large parasite reservoir circulating in asymptomatic carriers that might play an important role in the spread of resistance has previously been ignored. Difficulties in finding consistent associations may also have arisen because most of the studies primarily focused on single genes and markers (Djimdé *et al.*, 2001), rather than multiple markers in several gene loci, therefore neglecting mutations that are not directly associated with resistance, but compensate for fitness costs induced by resistance related mutations (Hastings & Donnelly, 2005; Warhurst, 2001). This is mainly because the most frequently applied molecular methods, such as PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) analysis or sequencing, are not suitable for the parallel analysis of multiple markers in large sample sets (Sangster *et al.*, 2002).

A monitoring approach on community level could give a more comprehensive assessment of the genetic profile of the parasite population circulating in a given area and contribute to a

broader understanding of the relationship between drug resistance genotypes and clinical failure rates as well as the dynamics and spread of drug resistant malaria.

Only a few studies used a community-based design for the determination of the genetic resistance background in parasites and its correlation with *in vivo* treatment response (Talisuna *et al.*, 2002a; Talisuna *et al.*, 2003a). However, whether the molecular profile of parasites circulating in the community matches the one observed among symptomatic patients seen at health facilities, has never been demonstrated.

We have recently developed a novel high throughput method based on DNA microarray technology which allows a parallel analysis of multiple SNPs in different genes in large sample sets (Crameri *et al.*, 2006, submitted, Chapter 3). Moreover, in a recent pilot study, we could demonstrate that there was no difference between the genetic drug resistance profile in clinical and community samples (Marfurt *et al.*, 2006, in preparation, Chapter 5).

It was the aim of the current study to investigate the role and applicability of the molecular drug resistance profiles in community samples for monitoring of drug resistant malaria. For this purpose, we conducted *in vivo* efficacy studies with the current first-line regimen amodiaquine (AQ) or chloroquine (CQ) plus sulphadoxine-pyrimethamine (SP) at different sites in Papua New Guinea (PNG). We established simultaneously the molecular drug resistance profile in blood samples collected in the communities from the catchment areas of the corresponding health facilities by using the new DNA microarray-based technology for the parallel analysis of antimalarial drug resistance markers. We then investigated the potential of molecular marker frequencies and genotype patterns to reflect the trends of clinical failure in different epidemiological settings and propose a model for surveillance of resistance based on the molecular resistance profile of the parasite.



## MATERIALS AND METHODS

### Study areas and design

*In vivo* drug efficacy studies and community-based cross-sectional surveys were conducted at three different sites in Papua New Guinea (PNG) between October 2002 and March 2005. These sites included 1) the Sigimaru health centre (HC) in the Karimui area (Simbu Province), 2) the Kunjingini HC in the South Wosera area (East Sepik Province), and 3) the Mugil HC in the North Coast area of Madang (Madang Province). In Karimui, a rural region in the highland fringe area of PNG, the studies were run between October and April in three consecutive years (2003, 2004, and 2005). In the Wosera, located in the floodplain of the Sepik river in the North-eastern part of the country bordering Indonesian Papua, the study period was between December and June in two following years (2003 and 2004). The study in the rainforest area at the North Coast of Madang was conducted between April 2004 and February 2005. Malaria transmission is perennial with limited variations between wet (October to April) and dry (May to September) season at all three sites. Transmission intensity decreases significantly with increasing altitude (Müller *et al.*, 2003) and is higher in the lowland regions of the Wosera and the North Coast than in the Karimui area, an elevated plateau situated at an altitude of 700 to 1200 m. Whereas there is little socioeconomic stratification between and within sites, with most of the inhabitants being subsistence farmers, there are differences with regard to health care provision and drug use patterns (Benet *et al.*, 2004; Genton *et al.*, 1995; Hii *et al.*, 2001; Mehlotra *et al.*, 2002; Müller *et al.*, 2004). Baseline characteristics of the study sites are summarized in table 1.

Scientific approval and ethical clearance for the study was obtained from the Medical Research and Advisory Committee (MRAC) of the Ministry of Health in PNG. Informed consent was first requested from all the communities involved and prior to recruitment, individual consent was obtained from each study participant and parents or legal guardians.

### Assessment of *in vivo* drug efficacy

Drug efficacy studies were conducted according to the standardised WHO protocol for low to moderate transmission areas (WHO, 2003) and are described in detail elsewhere (Marfurt *et al.*, 2006, submitted, Chapter 2). Children between 6 months and 7 years of age were enrolled

if they were presenting at the health centre with clinically overt and microscopically confirmed *P. falciparum* malaria and no danger signs for severe or complicated malaria (WHO, 2000) or signs of any other disease, malnutrition or anaemia. Standard AQ (for patients <14 kg) or CQ plus SP first line treatment (10 mg AQ or CQ per kg on Day 0, 1 and 2, and 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 0) was administered under supervision over the first three days. Visits for the follow-up were scheduled on Day 1, 2, 3, 7, 14, and 28. On every visit, patients were clinically examined and a Giemsa-stained blood slide was taken for the microscopic assessment of parasitaemia. A blood sample was taken on Day 0 (pre-treatment sample) and on Days 14 and 28 or any day of treatment failure for molecular genotyping purposes. At the end of the follow-up, the patients were classified according to their clinical and parasitological responses into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF), or adequate clinical and parasitological response (ACPR) (WHO, 2003). Distinction between recrudescence and new infection was accomplished by the comparison of the *msp2* genotyping patterns of Day 0 and treatment failure samples (Snounou & Beck, 1998) and failure rates were corrected accordingly (Marfurt *et al.*, 2006, submitted, Chapter 2).

### **Community-based cross-sectional surveys**

Cross-sectional surveys were conducted in the catchment areas of the health centres where *in vivo* studies were run using a randomized household approach. To obtain a representative random sample of the parasite population circulating in the corresponding communities (i.e., approximately 100 PCR-positive *P. falciparum* samples from each location), we collected between 300 and 350 blood specimens per community (Mehlotra *et al.*, 2002). Overall, a total of 2013 individuals from randomly selected villages and households were recruited for the community surveys between 2003 and 2005.

Apart from demographic characteristics, collected information included history of sickness (onset, type and duration of symptoms), health facility attendance, purchase or consumption of drugs outside health facilities, and antimalarial treatment courses received in the preceding year (extracted from health books when available). Axillary temperature was measured with an electronic thermometer and spleen size was assessed in the recumbent position using Hackett's grading system (Gilles & Warrell, 1993). Blood samples for parasitological examination by microscopy, haemoglobin (Hb) level determination (HemoCue®, Ångelholm,

Sweden), and molecular assessment of parasite genotypes were collected by venepuncture using 2 ml EDTA-Vacutainer™ tubes (BD Biosciences, Allschwil, Switzerland). Plasma was separated by centrifugation and red blood cell pellets were stored frozen until further processing.

### Molecular analyses

DNA was extracted using QIAamp® DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

Assessment of SNPs for drug resistant malaria in community samples was done for *pfmdr1* codons N86Y, Y184F, S1034C, N1042D and D1246Y, *pfcr1* codons K76T, H97Q, T152A, S163R, A220S, Q271E, N326D/S, I356L/T and R371I, *pfdhfr* codons A16V, N51I, C59R, S108N/T and I164L, *pfdhps* codons S436A, A437G, K540E, A581G, and A613T/S, and *pfATPase6* codons S538R, Q574P, A623E, N683K, and S769N. The method is based on parallel PCR amplification of the target sequences followed by primer extension mediated mini-sequencing using fluorochrome-labelled ddNTPs. Subsequent base calling occurs on a microarray upon sequence specific hybridisation (Crameri *et al.*, 2006, submitted, Chapter 3; Marfurt *et al.*, 2006, in preparation, Chapter 4). Multiplicity of infection (MOI) for each sample was assessed by determining the number of *msp2* genotypes (Felger and Beck, 2002).

### Statistical analyses

Data were double entered in EpiData software (version 3.02, Odense, Denmark) and analysis was performed using STATA software (version 8.2; Stata Corp., College Station, Texas).

To estimate the allele frequencies of resistance markers in our sample set, we used a non linear statistical model that takes into account the effects of varying multiplicity of infection and assumes that resistant and sensitive parasite clones are transmitted independently. The likelihood of a sample containing no resistant clones is  $(1 - p)^n$ , where  $p$  is the frequency for the mutant allele and  $n$  is the multiplicity of infection of the sample. Similarly, the likelihood for the sample to contain no wild-type allele is  $p^n$  and for a mixture of both, a wild-type and a resistant allele, is  $1 - p^n - (1 - p)^n$ . The likelihood over the whole data set for  $p$  is computed as

the product of this likelihood over all samples, using values of  $n$  derived from *msh2* genotyping results. A Markov Chain Monte Carlo algorithm (Program Winbugs 1.3) was used to obtain estimates of mutant allele frequencies and credible intervals (Bayesian confidence intervals (CI) for  $p$ ), making use of this likelihood, and assuming a uniform (0.1) prior distribution for  $p$  (Schneider *et al.*, 2002).

The significance of different proportions was determined by using  $\chi^2$  test for trend or Fisher's exact test as applicable and differences in allele frequencies were assessed by the comparison of 95% confidence intervals.

## RESULTS

### *In vivo* drug efficacy of AQ or CQ plus SP

Clinical and parasitological monitoring up to Day 28 between 2003 and 2005 at all three sites was accomplished for a study group of 649 children. CQ plus SP was given to 128 (19.7%) children (median age of 6 years), whereas 521 (80.3%) children were treated with AQ plus SP (median age of 4 years). PCR-corrected treatment failure rates up to Day 28 for *P. falciparum* at the three sites in the different years are shown table 2. In the Karimui area, treatment failure rates up to Day 28 decreased over the three-year period from 28% to 18% and 16%, respectively ( $\chi^2_{(2)}=4.81$ ,  $p=0.09$ ). In the South Wosera area, overall failure rate tended to increase from 16% in 2003 to 22% in 2004 ( $\chi^2_{(2)}=1.19$ ,  $p=0.28$ ). In 2004, treatment failure rate after PCR-adjustment was 11.5% in the North Coast area of Madang (Marfurt *et al.*, 2006, submitted, Chapter 2).

### Characteristics of the survey populations

Key characteristics of the random community sub-samples collected at the three sites between 2003 and 2005 are depicted in table 1. Whereas age distribution, sex ratio, and mean axillary temperature were similar between sites, mean haemoglobin levels were lower in the high transmission areas of the Wosera and the North Coast. Microscopic *P. falciparum* prevalence rates ranged between 13% and 27%, depending on the site and the respective year, and prevalence rates determined by a positive *msp2* PCR result were higher (between 20% and 41%, respectively). Though mean multiplicity of infection (MOI) was not significantly different between and within sites over time (range: 1.5 to 1.8), the proportion of multiple versus single infections varied between high and moderate transmission areas (data not shown).

### Mutant allele frequencies of drug resistance markers

From a total of 2013 collected blood samples, 695 (34.5%) were found to be *P. falciparum* positive by *msp2* PCR screening and subsequent mutation analyses were successfully accomplished for 633 (91%) of these samples. Polymorphisms were found in *pfmdr1* codons

N86Y, Y184F, and N1042D, *pfcr*t codons K76T, A220S, N326D, I356L, and S163R, *pfdhfr* codons C59R and S108N, and *pfdhps* codons A437G and K540E. None of the other 21 SNPs was detected as mutated allele in any of the infections analysed (Table 2).

Regarding CQ relevant markers in *pfcr*t, we measured high mutant allele frequencies (MAFs) for K76T (0.90 to 1.00), N326D (0.77 to 0.97), and I356L (0.76 to 0.98) at all three sites. Mutation rates of A220S were highest at the North Coast (0.95) and significantly lower in Karimui (0.48 to 0.63) and the South Wosera (0.61 to 0.81). Mutated *pfcr*t S163R alleles were rarely detected ( $\leq 0.01$ ) and at the latter two sites only. Whereas MAFs for *pfmdr1* N86Y reached quasi fixed levels in Karimui (0.99 to 1.00) and at the North Coast (0.93), frequencies were significantly lower in the South Wosera (0.69 to 0.72). *Pfmdr1* polymorphisms Y184F and N1042D were never detected in samples from Karimui, and frequencies were low (0.03 to 0.07) at the other two sites.

Regarding SP relevant markers, MAFs for *pfdhfr* S108N and C59R were high at all three sites (0.82 to 0.97 and 0.73 to 0.95, respectively), but were still moderate in *pfdhps* A437G (0 to 0.21) and very low in *pfdhps* K540E (0 to 0.03) (Table 2).

Looking at the dynamics over time at a single site, significant changes in MAFs were found in the Karimui area for *pfcr*t N326D and I356L, which decreased significantly between 2003 and 2005, and 2004 and 2005, respectively. By comparing the dynamics of molecular markers between Karimui and the South Wosera, we observed reverse trends over time for polymorphisms *pfcr*t A220S and *pfdhps* A437G. Whereas MAFs for these two gene loci decreased significantly between 2004 and 2005 in Karimui (0.63 to 0.48, and 0.10 to 0.04, respectively), they increased significantly between 2003 and 2004 (0.61 to 0.81, and 0 to 0.03, respectively) in the South Wosera (Table 2).

### Patterns of drug resistant genotypes

All patient isolates were coded according to presence or absence of mutant alleles and isolates showing both, wild-type and mutant allele, were treated as mutant. Likewise, infecting genotypes were coded according to the most highly mutated *pfmdr1*, *pfcr*t, and *pfdhfr/pfdhps* alleles present in the sample.

A single mutation N86Y was the predominant genotype in *pfmdr1* at all three sites (72% to 100%; Table 3). There was a significant difference in prevalence rates between the Karimui

and the Wosera areas in the years 2003 and 2004 (99% versus 71%, and 100% versus 71%, respectively,  $p < 0.001$  in both years). Though we observed a slightly decreasing trend in the prevalence of the *pfmdr1* wild-type allele in the Wosera over time ( $p = 0.43$ ), this was not accompanied by an increase of the single N86Y mutation ( $p = 0.97$ ), but the occurrence of genotypes with single or double mutations Y184F and N1042D.

Genotypes with a *pfcr1* wild-type, single K76T or double K76T+ N326D mutations were only observed in the Karimui and the Wosera areas, but they were not detected in any of the samples from the North Coast in Madang (Table 4). In this region, we only found genotypes with triple (K76T+N326D+N326D) or quadruple (+A220S) mutations. Prevalence rates of *pfcr1* triple and quadruple mutants were relatively stable in Karimui between 2003 and 2005 (32% to 36%, and 54 to 61%, respectively). This is in contrast to the observations in the Wosera, where quadruple mutations increased at the expense of triple mutations between 2003 and 2004, from 69% to 86% ( $p = 0.001$ ) and 15% to 4% ( $p = 0.002$ ), respectively.

For SP relevant genotypes (Table 5), the prevalence of fully wild-type *pfdhfr/pfdhps* alleles was low at all three sites (1 to 15%). The predominant genotype was the double *pfdhfr* mutant S108N+C59R, with prevalence rates between 56% and 93%. Whereas genotypes with the *pfdhps* A437G mutation (which always occurred in combination with the *pfdhfr* double mutation S108N+C59R) were detected in 7% to 22% of patient samples from the Karimui area, prevalence rates between 0% and 4% were lower at the other two sites. In Karimui, there was a significant decrease of this triple *pfdhfr/pfdhps* mutant between 2003 and 2005 ( $p = 0.002$ ), which was paralleled by a significant decrease of genotypes with single (S108N) and an increase in genotypes having the double (+C59R) mutation in *pfdhfr* ( $p = 0.003$  and  $0.001$ , respectively). A different longitudinal trend was observed in the Wosera area, where a significant increase of triple *pfdhfr/pfdhps* mutants between 2003 and 2004 ( $p < 0.001$ ) was accompanied by an increase of wild-type *pfdhfr/pfdhps* alleles ( $p < 0.001$ ) and a decrease of genotypes with the single and double mutations in *pfdhfr* ( $p = 0.38$  and  $0.01$ , respectively).

Because it was the aim to use the rate of SNPs within the population, we tested whether the genotype failure indices (GFIs), calculated as the ratio between prevalence of different genotypes to treatment failure rate, could predict the actual failure rate at a given site in the respective year. The trends in treatment failure rate over subsequent years (i.e., decreasing in Karimui and increasing in the Wosera) was best reflected by the GFI based on the prevalence of the combined *pfdhfr* S108N+C59R + *pfdhps* A437G genotype (Table 6).

## DISCUSSION

In the present study, we investigated the relationship between the genetic drug resistance background in parasite populations and clinical drug efficacy at three sites in Papua New Guinea (PNG). To clarify the question, whether the observed varying trends of *in vivo* drug efficacy between 2003 and 2005 in these regions reflected intrinsic variations or true dynamics of drug resistant malaria, we sought to explain these longitudinal *in vivo* trends by the molecular drug resistance profile assessed in the respective parasite populations. On an individual level, we had previously identified the triple mutation *pfdhfr* S108N+C59R plus *pfdhps* A437G to be a reliable predictor for treatment failure under the current first-line therapy with AQ or CQ plus SP (Marfurt *et al.*, 2006, in preparation, Chapter 4). Moreover, we could show that the genetic drug resistance profile of parasite populations circulating in community samples was representative for the profile detected in clinical samples which was indeed reflecting the corresponding *in vivo* failure rates (Marfurt *et al.*, 2006, in preparation, Chapter 5). We therefore used in the current studies a community-based sampling approach and applied a novel DNA microarray-based technology which enabled the parallel assessment of multiple single nucleotide polymorphisms (SNPs) in several genes in large sample sizes (Crameri *et al.*, 2006, submitted, Chapter 3).

After a long history of 4-aminoquinoline use, which was accompanied by accumulating reports of *in vivo* resistance to this drug class (Genton *et al.*, 2006; Müller *et al.*, 2003), treatment policy against uncomplicated malaria in PNG was changed to combination therapy with AQ or CQ plus SP in the year 2000. Only after two years of effective implementation, we measured treatment failure rates between 12% and 28%, with varying longitudinal trends depending on the area and the year (Table 2, Marfurt *et al.*, 2006, submitted, Chapter 2). The significant levels of parasitological *in vivo* resistance were reflected in the genetic drug resistance profile of the parasites. High mutant allele frequency (MAF) estimates were obtained not only for molecular markers for CQ resistance (CQR) in *pfprt* and *pfmdr1*, but also for important markers in *pfdhfr* and *pfdhps*, relevant for resistance to antifolates.

Whereas MAFs for key CQR markers *pfprt* K76T, N326D, and N326D reached almost fixed levels at all three sites, which was consistent with data from previous studies describing a highly mutated genetic CQR background in field isolates from PNG (Chen *et al.*, 2001; Mehlotra *et al.*, 2005; Nagesha *et al.*, 2003), MAFs between 0.48 and 0.95 for *pfprt* A220S varied considerably between sites. More importantly, MAFs in *pfprt* A220S, which proved to



be indicative for the presence of a highly CQ resistant *pfcr* quadruple mutant (K76T+N326D+N326D+A220S) in our studies, reflected the longitudinal trends of *in vivo* drug efficacy observed in Karimui and the Wosera. Whereas the decreasing *in vivo* trend in Karimui (28% in 2003, 18% in 2004, and 16% in 2005) was accompanied by MAFs in *pfcr* A220S of 0.57 in 2003 and 0.63 in 2004, which significantly decreased to 0.48 in 2005, the opposing *in vivo* trend in the Wosera (16% in 2003 and 22% in 2004) was reflected in a statistically significant increase in the frequency of the *pfcr* A220S mutation (0.61 in 2003 versus 0.81 in 2004). Though statistically not significant, a similar trend was observed when taking into account the complete *pfcr* genotype, with the prevalence of *pfcr* quadruple mutants decreasing between 2003 and 2005 from 60% to 54% in Karimui, and increasing between 2003 and 2004 from 69% to 86% in the Wosera.

Frequencies and genotype patterns of polymorphisms in *pfmdr1* (i.e., N86Y, Y184F and N1042D) were relatively stable over time in a given area, but prevalence rates for the single *pfmdr1* N86Y mutant of 99% to 100% in Karimui and 93% in the North Coast area of Madang were significantly higher than those of 71% observed in the Wosera. There is ample evidence from *in vitro* experiments that demonstrated single nucleotide polymorphisms (SNPs) in *pfmdr1* to confer resistance to CQ, but also altered sensitivity to other drug classes including quinine, arylaminoalcohols and artemisinin derivatives (Duraisingh *et al.*, 2000; Reed *et al.*, 2000; Sidhu *et al.*, 2005). Several lines of evidence, such as the lack of a straightforward associations between *pfmdr1* mutations and *in vivo* CQR (Basco & Ringwald, 1998; Haruki *et al.*, 1994; Pillai *et al.*, 2001; Pova *et al.*, 1998), frequently observed linkage disequilibrium between *pfcr* K76T and *pfmdr1* N86Y *in vivo* (Adagut *et al.*, 2001; Happi *et al.*, 2006, Mita *et al.*, 2006a), and the commonly accepted hypothesis that CQR is mediated by multigenic processes (Mu *et al.*, 2003), strongly suggest that SNPs in *pfmdr1* play an important role in modulating levels of CQR, but may not be sufficient indicators for *in vivo* CQR. Our data confirm that and also showed that *pfmdr1* N86Y was strongly linked with treatment failure on individual level when found in conjunction with the highly CQ resistant *pfcr* quadruple mutant (Marfurt *et al.*, 2006, in preparation, Chapter 4). However, this marker does not seem to be suitable for longitudinal monitoring of CQR on population level, the most likely reason being that this mutation is almost fixed in the parasite population. On the other hand, the observed differences in *pfmdr1* genotype patterns between sites (i.e., absence of *pfmdr1* polymorphisms Y184F and N1042D in Karimui) most likely resulted from different levels of previous quinine use in the highland versus the lowland areas. Most probably due to the fact that *P. falciparum*, the species responsible for severe malaria requiring treatment with

quinine under prior drug policy in PNG, has become the predominant species in the Karimui area only within the last two decades (Müller *et al.*, 2006).

Regarding SP relevant markers, high MAFs for *pfdhfr* S108N and C59R at all three sites (0.82 to 0.97 and 0.73 to 0.95, respectively) were consistent with the observation of parasites harbouring the corresponding *pfdhfr* double mutation being the predominant genotype at all three sites. Similarly high mutation rates for the same *dhfr* genotype (83% in 2002 and 86% in 2003) were recently reported by Mita and colleagues (2006b) in a study conducted in Wewak, the capital town of the East Sepik Province. These high prevalence rates measured after only a short time of SP introduction as part of the official first-line regimen in PNG may have arisen due to several reasons. These include the 1) use of pyrimethamine (in combination with CQ) in mass drug administration campaigns between the late 1960s and the early 1970s (Spencer, 1992), 2) sporadic use of SP as part of the second-line treatment (together with quinine) against severe and treatment failure malaria, and 3) frequent use of cotrimoxazole (trimethoprim-sulphamethoxazole) against bacterial infections which may have exerted additional selection pressure for resistance conferring mutations in *pfdhfr* and *pfdhps* (Iyer *et al.*, 2001). Though MAFs of polymorphic *pfdhps* K540E and A437G were relatively low (between 0 and 0.03, and 0 to 0.21, respectively), the longitudinal *in vivo* efficacy trends were best reflected by the latter mutation. Whereas the decreasing treatment failure rates in Karimui were paralleled by decreasing MAFs for *pfdhps* A437G (from 0.21 in 2003 to 0.04 in 2005), polymorphisms at this SNP site in the Wosera were absent in 2003 and started to emerge in 2004, which correlated with the increasing trend in treatment failure rates. The importance of the *pfdhps* A437G mutation in indicating clinical resistance were additionally confirmed by the observation that the trends in clinical response were not mirrored in the prevalence rates of *pfdhfr* genotypes alone, but were best reflected by prevalence rates of the genotypes having the triple *pfdhfr* (S108N+C59R) plus *pfdhps* (A437G) mutation.

Taken together, we describe here a highly mutated CQR background combined with *pfdhfr* mutations consistent with a moderately pyrimethamine resistant phenotype and the emergence of key mutations in *pfdhps*. Regarding the previously known high levels of *in vivo* CQR and reduced pyrimethamine sensitivity, significant levels of *in vivo* failure with the combination of AQ or CQ plus SP are not surprising. The combination of SP with a nearly 90% inefficacious partner drug was not expected to prolong the useful therapeutic life of the combination of these two drug classes (Watkins *et al.*, 2005). In fact, our *in vivo* and molecular data suggest that SP, and more specifically sulphadoxine, is the effective

component in the current first-line regimen and hence, molecular monitoring of resistance to this component is important under constant treatment policy in PNG.

It is a long-standing goal in malaria control to use molecular markers as a rapid means for the surveillance of resistance in order to provide timely and evidence-based information for policy formulation. Hence, there is a need for practical models to predict the risk for treatment failure based on molecular data collected in different epidemiological contexts using treatment regimens consisting of single or multiple drug classes. Determination of a genetic failure index (GFI), calculated as the ratio between the prevalence of mutated genotypes and treatment failure rate, has been proposed by several authors (Djimdé *et al.*, 2001; Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003). We tested the validity of this GFI for different CQR and SPR relevant genotypes and identified the GFI based on the combined *pflhfr/pfdhps* genotype to be the most suitable indicator for *in vivo* drug efficacy. These observations do not only further corroborate our previous findings showing this genotype to be the best predictor for treatment failure on individual level (Marfurt *et al.*, 2006, in preparation, Chapter 4), they also indicate this genotype to be a valuable marker for the level clinical failure on population level over time at a given site. Though our results are not consistent with African studies in Tanzania (Mugittu *et al.*, 2004; Mutabingwa *et al.*, 2001) and Ghana (Mockenhaupt *et al.*, 2005), which showed that the *pfdhps* genotype was not indicative for treatment failure with SP monotherapy, recent studies from Uganda (Dorsey *et al.*, 2004) and Laos (Berens *et al.*, 2003) reported *pfdhps* mutations to be important markers for unsuccessful treatment response to combination therapy with CQ plus SP. These conflicting reports underscore the need for a careful baseline assessment of the molecular marker profile in parasite populations, the investigation of its relationship with *in vivo* treatment response, and the monitoring of its dynamics over time. They clearly indicate that former drug history is an important determinant of the genetic resistance background in parasites and that SP resistance may emerge and spread very differently according to whether SP was used as monotherapy or introduced as partner component in a combination regimen.

However, when we compared GFIs with clinical failure rates between sites in 2004 (i.e., 22% in the Wosera, 18% in Karimui, and 12% at the North Coast of Madang), the corresponding absolute values of the GFI (i.e., 0.2 in the Wosera, 0.6 in Karimui, and 0.1 at the North Coast of Madang) did not reflect the respective *in vivo* efficacy trends. These findings further underscore the importance of drug pressure exerted on parasite populations in determining molecular resistance dynamics, especially in areas with low to moderate transmission intensity (Talisuna *et al.*, 2002b; Talisuna *et al.*, 2003b; White; 2004). Previous drug histories

were different at the three sites. The North Coast area had by far the highest CQ pressure due to good health care provision as well as easy access to drugs in the nearby town of Madang. In contrast, former 4-aminoquinoline use is assumed to be lower in the remote regions in Karimui and the Wosera. However, former drug pressure with antifolates in the course of previous mass drug administration campaigns has been higher in the latter two regions (McMahon, 1973; Spencer, 1992). Though parasite CQR at all three sites can be regarded as having reached a plateau level, resistance to SP evolved and spreads at a different speed and seems to be mainly dependent on both, former and present antifolate pressure, the latter being predominantly modulated by the level of implementation of the new drug policy.

In view of the commonly accepted hypothesis that increasing drug pressure leads to the emergence and spread of drug resistant genotypes, how can we explain the intriguing observation of opposing *in vivo* and molecular resistance trends in the Karimui and the Wosera areas? Again, we believe that these results clearly reflect the fact that other important parameters, such as drug use patterns as well as immunity related to transmission intensity (Djimdé *et al.*, 2003; Francis *et al.*, 2006), play a significant role in determining the level and spread of parasite resistance in a given area. In Karimui, the conduct of our studies lead to an increased awareness of malaria and resulted in an overall change in health seeking behaviour. At the same time, health care provision generally improved due to better medical supply to the remote highland area. Moreover, missionaries, who represented the main source of chloroquine provision outside health facilities in previous years, left the area in 2003. Taken together, all these factors may have resulted in an overall adequate drug pressure (i.e., correct dosage of combination therapy to a higher proportion of malaria cases) acting on a relatively small parasite population, therefore preventing the further development and spread of parasite resistance in this moderate transmission area (Talisuna *et al.*, 2002b; Talisuna *et al.*, 2006). The inverse happened in the Wosera area during the same time period. Inadequate drug pressure (i.e., indiscriminate monotherapy with AQ or CQ) exerted on a large and heterogeneous parasite population originated not only from health systems management problems in the area which lead to an undersupply with SP (local health extension officer at the Kunjingini health centre, personal communication). Also drug consumption outside health facilities was frequent (10% and 12% of the survey populations in 2003 and 2004, respectively) with AQ or CQ monotherapy mainly distributed by so-called ‘marasin meris’, community health workers who were inadequately trained by a local NGO. These factors may have further enhanced parasite CQR which consequently lead to reduced capacity of CQ to protect the development of resistance to SP.

In summary, we have shown that a comprehensive baseline assessment of resistance markers including the investigation of their relationship with treatment response is important for the identification of appropriate marker sets. We could demonstrate that the molecular drug resistance profile of parasite populations in community samples represented the profile in clinical samples and was capable to reflect the longitudinal *in vivo* drug efficacy trends observed at the respective health centres. However, our community-based molecular monitoring approach will have to be further evaluated in geographical areas at both extremes of transmission intensity and different drug use patterns (e.g., rural versus urban areas) in order to test its validity as complementary resistance monitoring tool. Furthermore, marker sets will have to be adapted to future monitoring purposes, such as the inclusion of markers for newly introduced or withdrawn drug classes. Our data confirm that the genetic drug resistance background of the parasite is only one of many factors determining clinical outcome and one which has evolved differently according to epidemiological characteristics as well as history and patterns of drug use in a given area. The frequently suggested surveillance indicator GFI, which is simply based on prevalence of mutated genotypes and treatment failure rate, does not seem to be comparably applicable in different epidemiological settings. Other proxy indicators for important determinants of antimalarial parasite as well as *in vivo* resistance, such as immunity, transmission intensity, and drug pressure, are necessary to be considered in future public health models for monitoring drug resistant malaria.

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**TABLE 1:** Characteristics of study sites and survey populations between 2003 and 2005

Study site	Karimui area			South Wosera area		North Coast area
Endemicity*	mesoendemic			mesoendemic		mesoendemic
Transmission intensity <sup>§</sup>	moderate			high		high
Year	2003	2004	2005	2003	2004	2004
Characteristics	n=265	n=347	n=359	n=317	n=366	n=359
Age (mean (95% CI, range), yrs)	18.9 (17.0-20.7, 0.5-60)	19.4 (17.7-21.2, 0.5-73)	15.4 (14.0-16.8, 0.5-66)	20.4 (18.7-22.2, 0.5-70)	21.3 (19.6-23.1, 0.5-69)	19.6 (17.8-21.5, 0.5-70)
Sex: females/n (%)	137/265 (51.7)	192/347 (55.3)	186/359 (51.9)	159/317 (50.1)	189/366 (51.6)	182/359 (50.7)
Temperature (mean (95% CI), °C)	36.1 (36.0-36.1)	36.1 (36.0-36.1)	36.4 (36.4-36.5)	36.6 (36.5-36.6)	36.5 (36.5-36.6)	36.4 (36.3-36.4)
Hb (mean (95% CI), g/dl)	11.5 (11.2-11.8)	12.1 (11.8-12.3)	11.4 (11.2-11.7)	10.7 (10.5-10.9)	10.7 (10.6-10.9)	10.4 (10.3-10.6)
<i>Pf</i> prevalence by <i>microscopy</i> (x/n (%), (95% CI))	34/258 (13.2, 9.3-17.9)	64/346 (18.5, 14.5-23.0)	82/358 (22.9, 18.7-27.6)	55/314 (17.5, 13.5-22.2)	96/356 (27.0, 22.4-31.9)	82/358 (22.9, 18.7-27.6)
<i>Pf</i> prevalence by <i>msp2</i> nPCR (x/n (%), (95% CI))	102/263 (38.8, 32.9-45.0)	71/347 (20.5, 16.3-25.1)	131/359 (36.5, 31.5-41.7)	129/317 (40.7, 35.2-46.3)	147/366 (40.2, 35.1-45.4)	115/359 (32.0, 27.2-37.1)
Mean multiplicity of infection (MOI) (95% CI, range)	1.46 (1.32-1.60, 1-4)	1.59 (1.38-1.80, 1-4)	1.77 (1.62-1.92, 1-4)	1.77 (1.57-1.96, 1-6)	1.85 (1.68-2.02, 1-5)	1.54 (1.38-1.70, 1-5)
* <i>Pf</i> prevalence age group 2-9 (x/n (%), (95% CI))	13/93 (14.0, 7.7-22.8)	20/107 (18.7, 11.8-27.4)	44/138 (31.9, 24.2-40.4)	21/98 (23.6, 15.2-33.8)	36/110 (32.7, 24.1-42.3)	46/128 (35.9, 27.7-44.9)
* Spleen rate age group 2-9 (%), (95% CI))	nd	17.65 (10.23-27.43)	26.85 (18.78-36.24)	17.65 (10.23-27.43)	17.27 (10.73-25.65)	41.13 (32.37-50.32)

\* determined according to *P. falciparum* prevalence and spleen rates (i.e., proportion of individuals with enlarged spleen) of 11-50% in children aged 2-9 years (WHO, 2003); § (Benet *et al.*, 2004; Hii *et al.*, 2001; Müller *et al.*, 2004), n, total number of people surveyed; CI, confidence interval; Hb, haemoglobin; *Pf*, *P. falciparum*; *msp2*, merozoite surface protein 2; nPCR, nested polymerase chain reaction; nd, not determined

**TABLE 2:** Maximum likelihood estimates of mutant allele frequencies of polymorphic gene loci in *pfmdr1*, *pfcr1*, *pfdhfr* and *pfdhps* from all three sites assessed between 2003 and 2005

Site	Karimui area (Simbu Province)						South Wosera (East Sepik Province)						North Coast (Madang Province)		
Year	2003			2004			2003			2004			2004		
TFR (%)	27.8			18.3			16.1			21.7			11.5		
SNP*	n	MAF	95% CI	n	MAF	95% CI	n	MAF	95% CI	n	MAF	95% CI	n	MAF	95% CI
<i>pfmdr1</i> N86Y	93	<b>0.99</b>	(0.96, 1.00)	70	<b>1.00</b>		128	<b>1.00</b>		115	<b>0.72</b>	(0.65, 0.78)	139	<b>0.69</b>	(0.63, 0.75)
<i>pfmdr1</i> Y184F	93	<b>0.00</b>		70	<b>0.00</b>		127	<b>0.00</b>		115	<b>0.07</b>	(0.04, 0.10)	139	<b>0.06</b>	(0.04, 0.10)
<i>pfmdr1</i> N1042D	93	<b>0.00</b>		70	<b>0.00</b>		128	<b>0.00</b>		115	<b>0.04</b>	(0.02, 0.07)	140	<b>0.06</b>	(0.04, 0.10)
<i>pfcr1</i> K76T	93	<b>0.97</b>	(0.93, 0.99)	70	<b>1.00</b>		128	<b>0.93</b>	(0.89, 0.96)	115	<b>0.94</b>	(0.90, 0.96)	139	<b>0.90</b>	(0.86, 0.93)
<i>pfcr1</i> S163R	93	<b>0.01</b>	(0.00, 0.04)	69	<b>0.00</b>		125	<b>0.00</b>		115	<b>0.00</b>		137	<b>0.00</b>	
<i>pfcr1</i> A220S	93	<b>0.57</b>	(0.49, 0.65)	70	<b>0.63</b>	(0.54, 0.71)	127	<b>0.48</b>	(0.44, 0.50)	115	<b>0.61</b>	(0.45, 0.76)	140	<b>0.81</b>	(0.77, 0.86)
<i>pfcr1</i> N326D	93	<b>0.92</b>	(0.87, 0.96)	70	<b>nd</b>		127	<b>0.77</b>	(0.71, 0.83)	115	<b>0.82</b>	(0.76, 0.87)	139	<b>nd</b>	
<i>pfcr1</i> I356L	93	<b>0.94</b>	(0.89, 0.97)	70	<b>0.91</b>	(0.85, 0.96)	127	<b>0.76</b>	(0.70, 0.82)	115	<b>0.83</b>	(0.77, 0.87)	139	<b>nd</b>	
<i>pfdhfr</i> S108N	93	<b>0.83</b>	(0.77, 0.89)	69	<b>0.91</b>	(0.85, 0.96)	128	<b>0.94</b>	(0.91, 0.97)	115	<b>0.82</b>	(0.76, 0.87)	139	<b>0.86</b>	(0.82, 0.90)
<i>pfdhfr</i> C59R	93	<b>0.73</b>	(0.65, 0.80)	69	<b>0.91</b>	(0.85, 0.96)	128	<b>0.93</b>	(0.89, 0.96)	115	<b>0.75</b>	(0.68, 0.81)	139	<b>0.73</b>	(0.67, 0.78)
<i>pfdhps</i> A437G	93	<b>0.21</b>	(0.15, 0.28)	68	<b>0.10</b>	(0.05, 0.16)	128	<b>0.04</b>	(0.04, 0.04)	115	<b>0.00</b>		139	<b>0.03</b>	(0.02, 0.06)
<i>pfdhps</i> K540E	93	<b>0.01</b>	(0.00, 0.05)	68	<b>0.00</b>		128	<b>0.01</b>	(0.00, 0.03)	115	<b>0.00</b>		139	<b>0.01</b>	(0.00, 0.03)

TFR, treatment failure rate; SNP, single nucleotide polymorphism; n, number of samples analysed; MAF, mutant allele frequency; CI, Bayesian confidence intervals for maximum likelihood estimates of MAF; nd, not determined; dark grey shading, significant increase in MAF between respective years; bright grey shading, significant decrease in MAF between respective years; \* To simplify matters, only polymorphic loci are depicted in the table (i.e., all other gene loci analysed were never found as mutated allele)

**TABLE 3:** *pfmdr1* genotype patterns assessed in community samples from all three sites between 2003 and 2005

<i>pfmdr1</i> SNPs	Karimui area Simbu Province				South Wosera area East Sepik Province						North Coast area Madang Province			
N86Y														
Y184F														
N1042D														
Year	Treatment failure rates													
2003	27.8%			16.1%										
x/n	1/94	93/94	21/115	82/115	2/115	2/115	6/115	2/115						
%	1.1	99.0	18.3	71.3	1.7	1.7	5.2	1.7						
2004	18.3%			21.7%								11.5%		
x/n	67/67			20/137	98/137	3/137	2/137	7/137	5/137	2/137	3/95	88/95	2/95	2/95
%	100.0			14.6	71.5	2.2	1.5	5.1	3.7	1.5	3.2	92.6	2.1	2.1
2005	16.4%													
x/n	125/125													
%	100.0													

*pfmdr1*, *Plasmodium falciparum* multi drug resistance gene 1; white box, wild-type allele; shaded box, mutated allele; SNP, single nucleotide polymorphism; x, number of samples detected with a given genotype; n, total number of samples analysed in the respective site and year; (genotype patterns detected in one of all 633 samples only are not included in table 3)

**TABLE 4:** *pfcrt* genotype patterns assessed in community samples from all three sites between 2003 and 2005

<i>pfcrt</i> SNPs	Karimui area Simbu Province							South Wosera area East Sepik Province						North Coast area Madang Province	
K76T															
I356L															
N326D															
A220S															
Year	Treatment failure rates														
2003	27.8%							16.1%							
x/n	2/94	2/94	1//94	1/94	29/94	1/94	56/94	9/115	7/115	1/115	2/115	17/115	79/115		
%	2.1	2.1	1.1	1.1	30.9	1.1	59.6	7.8	6.1	0.9	1.7	14.8	68.7		
2004	18.3%							21.7%						11.5%	
x/n		1/67	2/67		23/67		41/67	13/137				5/137	118/137	3/95	91/95
%		1.5	3.0		34.3		61.2	9.5				3.7	86.1	3.2	95.8
2005	16.4%														
x/n	8/125		1/125	1/125	44/125	1/125	68/125								
%	6.4		0.8	0.8	35.2	0.8	54.4								

*pfcrt*, *Plasmodium falciparum* chloroquine transporter gene; white box, wild-type allele; shaded box, mutated allele; SNP, single nucleotide polymorphism; x, number of samples detected with a given genotype; n, total number of samples analysed in the respective site and year; (genotype patterns detected in one of all 633 samples only are not included in table 4)



**TABLE 5:** *pf dhfr/pf dhps* genotype patterns assessed in community samples from all three sites between 2003 and 2005

<i>pf dhfr/dhps</i> SNPs	Karimui area Simbu Province					South Wosera area East Sepik Province				North Coast area Madang Province				
S108N														
C59R														
A437G														
K540E														
Year	Treatment failure rates													
2003	27.8%					16.1%								
x/n	7/94	1/94	11/94	53/94	20/94	2/115	14/115	99/115						
%	7.5	1.1	11.7	56.4	21.3	1.7	12.2	86.1						
2004	18.3%					21.7%				11.5%				
x/n	5/67			55/67	7/67	20/137	12/137	99/137	5/137	2/95	1/95	88/95	1/95	3/95
%	7.5			82.1	10.5	14.6	8.8	72.3	3.7	2.1	1.1	92.6	1.1	3.2
2005	16.4%													
x/n	3/125		2/125	111/125	9/125									
%	2.4		1.6	88.8	7.2									

*pf dhfr*, *Plasmodium falciparum* dihydrofolate reductase; *pf dhps*, *Plasmodium falciparum* dihydropteroate synthase; white box, wild-type allele; shaded box, mutated allele; SNP, single nucleotide polymorphism; x, number of samples detected with a given genotype; n, total number of samples analysed in the respective site and year; (genotype patterns detected in one of all 633 samples only are not included in table 5)

**TABLE 6:** Genotype failure indices for combined *dhfr/dhps* genotypes for all three sites between 2003 and 2005

Year	Site	Karimui Simbu Province		South Wosera East Sepik Province		North Coast Madang Province	
		P	GFI*	P	GFI	P	GFI
<b>2003</b>	<b>TFR</b>	<b>27.8%</b>		<b>16.1%</b>			
	G1: <i>pfldhfr</i> S108N+C59R + <i>pfldhps</i> A437G	21.3	<b>0.8</b>	0.0	<b>0.0</b>		
	G2: <i>pfldhfr</i> S108N+C59R	56.4	<b>2.0</b>	86.1	<b>5.3</b>		
<b>2004</b>	<b>TFR</b>	<b>18.3%</b>		<b>21.7%</b>		<b>11.5%</b>	
	G1: <i>pfldhfr</i> S108N+C59R + <i>pfldhps</i> A437G	10.4	<b>0.6</b>	3.6	<b>0.2</b>	1.1	<b>0.1</b>
	G2: <i>pfldhfr</i> S108N+C59R	82.1	<b>4.5</b>	72.3	<b>3.3</b>	92.6	<b>8.0</b>
<b>2005</b>	<b>TFR</b>	<b>16.4%</b>					
	G1: <i>pfldhfr</i> S108N+C59R + <i>pfldhps</i> A437G	7.2	<b>0.4</b>				
	G2: <i>pfldhfr</i> S108N+C59R	88.8	<b>5.4</b>				

P, prevalence; GFI, genotype failure index; \* GFIs are calculated as the ratio between prevalence of mutated genotype to treatment failure rate at a given site in the respective year; TFR, treatment failure rate; G, genotype (GFIs are shown for the two predominant *dhfr/dhps* genotypes only)

## Chapter 7

### **The association of mutations in *Plasmodium vivax* *dhfr* and *mdr1* and *in vivo* resistance to amodiaquine or chloroquine plus sulphadoxine-pyrimethamine in Papua New Guinea**

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## ABSTRACT

**BACKGROUND** Molecular mechanisms and markers for sulphadoxine-pyrimethamine (SP) resistance in *P. vivax* have been reported. However, molecular correlates involved in resistance to 4-aminoquinolines and data on their relationship with *in vivo* treatment response are still scarce.

**METHODS** We assessed *P. vivax dhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *mdr1* (Y976F and F1076L) mutations in pre-treatment samples from 104 patients with a *P. vivax* mono-infection who received amodiaquine (AQ) or chloroquine (CQ) in combination with SP in Papua New Guinea (PNG) and investigated the association between infecting genotype and treatment response.

**RESULTS** Treatment failure rate reached 13% with the new combination regimen. Polymorphisms in *pvdhfr* codons F57L/I, S58R, T61M, S117T/N, and *pvmldr1* codon Y976F were detected in 61%, 68%, 21%, 41%, and 39% of the samples, respectively. Taken independently, the single mutant *pvdhfr* 57 showed the strongest association with treatment failure (OR=9.31,  $p=0.01$ ). Presence of the quadruple mutant *pvdhfr* 57L+58R+61M+117T with *pvmldr1* mutation 976F best predicted treatment failure (OR=10.25,  $p<0.01$ ). The difference in failure rates between sites was reflected in the genetic drug resistance profile of the respective parasite populations.

**CONCLUSIONS** Our study identified a novel molecular marker in *pvmldr1* to be associated with *in vivo* response to AQ or CQ plus SP. Our results suggest *pvdhfr* F57L/I, T61M, and S117T/N plus *pvmldr1* Y976F as a suitable marker set for the molecular monitoring of *P. vivax* resistance against the current first-line therapy in PNG.

## INTRODUCTION

More than 50 % of all malaria cases outside Africa are caused by *Plasmodium vivax* and an estimated number of 70-80 million people are infected each year (Mendis *et al.*, 2001). *Plasmodium vivax* has considerable clinical and socioeconomic impact in endemic countries and resurgence of the disease is mainly attributable to the emergence of parasite resistance to the commonly available and applied therapies (Baird, 2004; Sattabongkot *et al.*, 2004). Moreover, research efforts lagged behind those for *P. falciparum* because of the lack of a continuous *in vivo* culture system and field studies being hampered by generally low parasitaemias in natural infections.

Chloroquine (CQ) resistant *P. vivax* was first described in Papua New Guinea (PNG) in 1989 (Whitby *et al.*, 1989) and thereafter, reduced CQ sensitivity of *P. vivax* has been reported from several endemic countries including Indonesia (Baird *et al.*, 1991; Schwartz *et al.*, 1991), Thailand and Myanmar (Myat *et al.*, 1993; Tan-ariya *et al.*, 1995), the Indian subcontinent (Dua *et al.*, 1996; Garg *et al.*, 1995), and South America (Garavelli *et al.*, 1996; Soto *et al.*, 2001). Because it has long been thought that antifolates, such as the fixed dose combination sulphadoxine-pyrimethamine (SP), are less active against *P. vivax*, an assumption which was mainly based on clinical studies failing to demonstrate SP efficacy against this species (Young & Burgess, 1959), SP has never been recommended for *P. vivax* malaria. Nevertheless, increasing levels of resistance of *P. falciparum* to CQ led to the adoption of SP as a cheap and safe alternative first-line-line option in many countries in South East Asia, Central and South America and Oceania, where both species are endemic, and *P. vivax* resistance to SP had developed rapidly in many areas within only a few years after its initial deployment as monotherapy (Baird, 2004; Peters, 1998; Pukrittayakamee *et al.*, 2000).

Hypnozoites, the latent liver stages of *P. vivax*, can give rise to a recurrent intra-erythrocytic infection between 3 weeks and several months after the initial infection depending on the strain. Therefore, *in vivo* assessment of drug efficacy is complicated by difficulties to clearly differentiate between treatment failures (true recrudescences originating from asexual blood stage parasites), relapses (red blood cell infection originating from hypnozoites) and newly acquired infections. Moreover, comparison of data is aggravated by the lack of studies following standardised protocols and classifications. Therefore, as in the case of *P. falciparum*, the assessment of molecular drug resistance markers could be a valuable complementary tool for the mapping and regular monitoring of drug resistant *P. vivax* malaria

(Plowe, 2003; WHO, 2006). Several genes related to resistance to the commonly used drugs have been described in *P. falciparum*. Orthologous genes of *pfdhfr* (*Plasmodium falciparum* dihydrofolate reductase), *pfdhps* (dihydropteroate synthase), *pfcr* (chloroquine resistance transporter gene) and *pfmdr1* (multidrug resistance gene 1) have been found in *P. vivax*, notably *pvdhfr* (Eldin de Pécoulas *et al.*, 1998a), *pvdhps* (Korsinczky *et al.*, 2004), *pvcg10* (Nomura *et al.*, 2001) and *pvmr1* (Brega *et al.*, 2005), respectively. Whereas no evidence could be found for an association between point mutations in both, *pvcg10* and *pvmr1*, and CQ resistance in *P. vivax* field isolates (Nomura *et al.*, 2001; Sà *et al.*, 2005), there are several laboratory studies which have clearly shown that pyrimethamine resistance is associated with a specific SNP accumulation in *pvdhfr* which leads to reduced enzyme affinity to the drug and corresponds to reduced sensitivity to pyrimethamine *in vitro* (Eldin de Pécoulas *et al.*, 1998b; Hastings & Sibley, 2002; Hastings *et al.*, 2005; Leartsakulpanich *et al.*, 2002; Tahar *et al.*, 2001). Furthermore, this association could be confirmed in epidemiological studies investigating the relationship of the genetic *pvdhfr* background and *in vivo* response to antifolates (Hastings *et al.*, 2004; Imwong *et al.*, 2001; Tjitra *et al.*, 2002). Likewise, reduced *in vitro* sensitivity to sulphadoxine (Chotinavich *et al.*, 2004; Russell *et al.*, 2003) and a relationship with clinical response to SP have been shown to be associated with SNPs in *pvdhps* (Imwong *et al.*, 2005; Korsinczky *et al.*, 2004).

In PNG, where all four *Plasmodium* species that infect humans are found, the majority of infections are caused by *P. falciparum* and *P. vivax* and mixed infections are common (Müller *et al.*, 2003). Antimalarial treatment in most health facilities in PNG is given based on presumptive clinical diagnosis without differentiation of the infecting species. Hence, after a long history of 4-aminoquinoline use against malaria, reports about reduced *in vivo* efficacy against *P. falciparum* and *P. vivax* malaria started to accumulate since the mid 1970s and the 1980s, respectively (Grimmond *et al.*, 1976; Rieckmann *et al.*, 1989), which reached unacceptably high levels in both species in the 1990s (Al Yaman *et al.*, 1996; Genton *et al.*, 2006). Despite the low use of SP in the country (only in combination with quinine against severe and treatment failure malaria), *P. falciparum* resistance to SP as well as reduced efficacy of SP against *P. vivax* has been described in the Madang province (Al Yaman *et al.*, 1994; Darlow *et al.*, 1982a; Darlow *et al.*, 1982b; Lamont & Darlow, 1982). First-line policy against uncomplicated malaria in PNG was changed to the combination of AQ or CQ plus SP, since this regimen showed initially satisfactory results against *P. falciparum* malaria (Jayatilaka *et al.*, 2003). In order to assess the status of the clinical efficacy of the current first-line regimen against *P. vivax* malaria in PNG after its effective implementation in the

year 2000, we conducted *in vivo* drug efficacy studies in three different areas in the country between 2004 and 2005 using standard clinical classifications according to the revised World Health Organization (WHO) protocol (Marfurt *et al.* 2006, submitted, Chapter 2; WHO, 2003). In the current study, we assessed *P. vivax* mutations in pre-treatment samples from patients with a monoinfection who received amodiaquine (AQ) or chloroquine (CQ) in combination with SP in PNG and investigated the association between infecting genotype and treatment response.

## SUBJECTS, MATERIALS, AND METHODS

### Subjects and therapeutic classification

*In vivo* drug efficacy studies were conducted between October 2004 and April 2005 in the Karimui area (Simbu Province), the South Wosera area (East Sepik Province), and the North Coast area of Madang (Madang Province) as described in detail elsewhere (Marfurt *et al.*, 2006, submitted, Chapter 2). Children between 6 months and 7 years of age were enrolled if they were presenting at the health centre with a clinically overt (axillary temperature  $\geq 37.5^{\circ}\text{C}$  or history of fever during the last 48 hours) and microscopically confirmed monoinfection with *P. vivax* (density  $>250$  asexual parasites per microlitre of blood). Further inclusion criteria were the absence of danger signs for severe or complicated malaria (WHO, 2000), signs of any other disease, malnutrition or anaemia. Standard AQ or CQ plus SP first-line treatment (10 mg chloroquine or amodiaquine per kg on Day 0, 1 and 2, and 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 0) was administered under supervision over the first three days. Follow-up visits were done on Day 1, 2, 3, 7, 14, and 28. On every visit, patients were clinically examined and a Giemsa-stained blood slide was taken for the microscopic assessment of parasitaemia.

Patients were advised to come to the health centre on any day if symptoms occurred. Whenever a child was diagnosed as treatment failure, standard second line treatment (5 mg artesunate per kg on Day 1 followed by 2.5 mg artesunate per kg on Day 2 to 7, and a single dose of 25 mg sulphadoxine per kg plus 1.25 mg pyrimethamine per kg on Day 3) was given.

Patients were classified as treatment failure (TF) when 1) clinical deterioration in the presence of *P. vivax* parasitaemia, or 2) parasitaemia between Day 3 and Day 28 with axillary temperature  $\geq 37.5^{\circ}\text{C}$ , or 3) parasitaemia between Day 7 and Day 28, irrespective of clinical conditions, was observed (WHO, 2001). Patients without clinical signs and without recurrent asexual parasites up to Day 28 were classified as adequate clinical and parasitological response (ACPR).



## Laboratory analyses

Finger prick blood samples were collected into EDTA Microtainer<sup>®</sup> tubes and DNA was extracted by using QIAamp<sup>®</sup> DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

Detection of single nucleotide polymorphisms in *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmr1* (Y976F and F1076L) was carried out with a LightCycler<sup>®</sup> system using FRET technology. Primers and probes were designed and synthesized by TIB<sup>®</sup> MOLBIOL (DNA synthesis service, Berlin, Germany) to detect five mutations in *pvdhfr* and two mutations in *pvmr1*. The sequences of the primers and probes are listed in table 1. The PCR mixture (20 µl) contained 5 µl of DNA template, probes (0.2 µM), primers (0.5 µM), MgCl<sub>2</sub> (3 mM) and 2 µl of FastStart DNA Master Hybridization probes (Roche Molecular Biochemicals). For *pvdhfr* codons, the PCR program included 40 cycles of denaturation at 95°C for 10 s, annealing of the primers and probes at 60°C for 10 s, and extension at 72°C for 20 s. The temperature change rates were 20°C/s for denaturation and annealing and 1°C/s for extension. Fluorescence was measured at channel F2 of the LightCycler<sup>®</sup> system at the end of the annealing phase of each cycle. For *pvmr1* codons, PCR program was similar except that amplification was performed with 45 cycles. The melting curve phase, period added after the PCR, consisted of one cycle of 95°C for 2 s, 40°C (*pvdhfr*) or 30°C (*pvmr1*) for 20 s, and heating at 80°C for 0 s. The temperature change rates were 20°C/s except for the final step which had a temperature transition rate of 0.1°C/s. Fluorescence was measured in continuous during the final step to determine a specific melting temperature of each genotype (Brega *et al.*, 2004; Brega *et al.*, 2005). A multi-clonal population could be identified by the presence of the two peaks simultaneously. DNA sequencing was used to confirm the presence of codon L or I at position 57 in *pvdhfr*.

## Statistics

Statistical analyses were performed by the use of STATA software (version 8.2; Stata Corp., College Station, Texas). The strength of association was evaluated by calculating odds ratios (OR). We used  $\chi^2$  test or Fisher's exact test as applicable and logistical regression analysis to assess the relationship between single or multiple mutations and treatment failure, taking into account other explanatory variables known to be associated with treatment outcome.

## RESULTS

### *In vivo* response to AQ or CQ plus SP

Baseline characteristics of all the children with a *P. vivax* monoinfection on admission day are depicted in table 2. AQ plus SP was given to 98 (94.2%) children, CQ plus SP to 5 (4.8%) children, and one (1.0%) was treated with primaquine plus SP. *P. vivax* treatment failure, defined as recurrent parasitaemia after Day 3 up to Day 28, irrespective of clinical symptoms (WHO, 2001), was seen in 13 (12.5%) of all children (Table 2). The vast majority of cases (11/13=84.6%) were parasitological failures at Day 28, one patient failed at day 6 and one failed at day 14. There was a significant difference of failure rates between sites ( $\chi^2_{(2)}=13.95$ ,  $p=0.001$ ): 10/34 (29.4%) *P. vivax* infections in the North Coast area of Madang and 3/46 (6.5%) in the Karimui area failed treatment, whereas all 27 infections were successfully cleared in the Wosera area (for details: Marfurt *et al.*, 2006, submitted, Chapter 2).

### Prevalence and relationship of *pvdhfr* and *pvmdr1* mutations

Mutation analyses were successfully accomplished in 100 (96.2%) of all pre-treatment samples from all the three study sites. Polymorphisms in *pvdhfr* codons F57L/I, S58R, T61M, S117T/N, and *pvmdr1* codon Y976F were detected in 61%, 68%, 21%, 41%, and 39% of samples, respectively (Figure 1). Depending on the codon position, a pure mutant allele was found in most of the samples (15% to 59%), whereas in 6% to 16% of the samples, a mutant allele was found in conjunction with the wild-type allele. None of the other SNPs (i.e., *pvdhfr* I173F/L and *pvmdr1* F1076L) was detected as mutated allele in any of the samples analysed. In all pre-treatment samples, nine different *pvdhfr* alleles were observed in single clone infections (78%), with the wild-type 57F+58S+61T+117S, the double mutant 57L+58R, and the quadruple mutant 57L+58R+61M+117L/I being the most prevalent haplotypes (28%, 25%, and 15%, respectively). Twenty-two of all samples contained mixed alleles at varying codon positions indicating polyclonal infections (Table 3). It is worth mentioning that the mutation F57L/I was always linked to S58R, and the T61M mutation always linked to the triple mutation F57L/I+S58R+S117T. Furthermore, in contrast to S117T, which was found in single, double, triple and quadruple mutations in *pvdhfr*, S117N was only observed in relation to single or double mutations.

### Association between *pvdhfr* and *pvmdr1* alleles and treatment outcome

To maximize our sample size, we pooled the data from all three study sites and evaluated the association between infections with single and combined mutant alleles in *pvdhfr* and *pvmdr1* and response to treatment. All patient isolates were coded according to presence or absence of mutant alleles and isolates showing both, wild-type and mutant allele, were treated as mutant. Likewise, infecting genotypes were coded according to the most highly mutated *pvdhfr* and *pvmdr1* alleles present in the sample.

In our study, explanatory variables such as fever, parasite density at day of enrolment or the combination regimen (SP plus CQ or AQ, respectively), were not associated with an increased risk of *P. vivax* treatment failure. However, risk of failure tended to decrease with increasing age (OR=0.60; 95% CI=0.36-1.00,  $p=0.05$ ). Regarding single molecular markers in *pvdhfr*, the presence of mutated codon positions 57 (either 57L or 57I), 58R, 61M and 117T were independently associated with an increased risk of treatment failure (Table 4). The same was observed with infections harbouring the mutation *pvmdr1* 976F. This relationship was further confirmed by the observation of a significant association between infections containing the wild-type allele 976Y and a positive treatment response (OR=0.18, 95% CI=0.04-0.74,  $p<0.01$ ).

In a further step, we established the *pvdhfr/pvmdr1* genotypes (i.e., combination of mutated alleles in both genes) of parasites for each patient sample. With regard to mutated gene loci, we could discriminate between 14 different genotypes (Table 5). Among those, seven were observed in treatment failure cases, whereas the remaining seven were exclusively found in patients with an adequate treatment response. Regarding *pvdhfr* genotypes alone, the risk of treatment failure was clearly related to the numbers of mutations present in an infection (OR=1.86, 95% CI=1.15-3.01,  $p=0.01$ ). However, the only significant association with a negative treatment outcome was seen with infecting genotypes having *pvdhfr* quadruple mutations combined with the *pvmdr1* mutation 976F (OR=10.25, 95% CI=2.44-43.11,  $p<0.01$ ).

Since treatment failure rate in the North Coast area (33%) was significantly higher than those of the Karimui area (7%) and the Wosera (0%), we investigated whether this difference in treatment outcome was reflected in the corresponding drug resistance marker profile of the parasite populations in the different sites (Table 6). Regarding polymorphisms in *pvdhfr*, there was a marked difference between sites for the mutated positions 57L ( $p(\chi^2_{(2)})=0.01$ ) and 58R ( $p(\chi^2_{(2)})=0.01$ ). Similarly, when compared with the two sites with lower treatment failure

rates, the prevalence of the mutated locus *pvmdr1* 976F was significantly higher in the North Coast area ( $p<0.001$ ). Correspondingly, the prevalence of the wild-type allele 976Y was lowest at this site with the highest level of *in vivo* resistance ( $p=0.001$ ). A similar picture was observed when frequencies of genotypes were compared.

The different levels of treatment response were not only reflected in a varying prevalence of the number of mutations in *pvdhfr* ( $p(\chi^2_{(2)})=0.03$ ). There was a significant difference between sites in the prevalence of the wild-type *pvdhfr/pvmdr1* genotype, which showed an increasing trend with decreasing treatment failure rate ( $p(\chi^2_{(2)})=0.03$ ). The inverse trend was observed with the two genotypes having a mutated *pvmdr1* 976F combined with the *pvdhfr* double (57L+58R) or quadruple (57L+58R+61M+117T) mutation, where prevalences were increasing with increasing levels of *in vivo* failure rates ( $p(\chi^2_{(2)})=0.001$  and 0.01, respectively; data not shown).

## DISCUSSION

Only four years after its effective implementation in PNG, the efficacy of the new first-line regimen of AQ or CQ plus SP against uncomplicated malaria has reached unacceptably low levels in both species (Marfurt *et al.*, 2006, submitted, Chapter 2). In this study, we investigated the relationship between drug resistance markers in *pvdhfr* and *pvm-dr1* in pre-treatment samples from patients with a *P. vivax* monoinfection and therapeutic outcome with the newly introduced combination regimen. We measured high prevalence rates of mutated key markers in both genes and demonstrated an association between infecting *pvdhfr/pvm-dr1* genotype and *in vivo* treatment response. Furthermore, different levels of treatment failure rates observed at different study sites were reflected in the genetic drug resistance profile of the corresponding parasite populations. This finding is probably the most important one since it validates the usefulness of molecular markers to monitor *P. vivax* resistance to antimalarial drugs in order to aid policy makers to develop rationale treatment strategies.

Regarding all single nucleotide polymorphisms analysed in *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvm-dr1* (Y976F and F1076L), we found a high prevalence of infections harbouring parasites with mutated gene loci (i.e., between 20% and 70%, depending on the locus analysed). Furthermore, we observed a high degree of diversity of different *pvdhfr* genotypes in our sample set deriving from three different areas within the same country. In most of the samples, we detected *pvdhfr* wild-type alleles (28%), double (34%), and quadruple mutations (21%), whereas the rate of single and triple mutations were lower (6% and 11%, respectively). Among the genotypes having double and quadruple mutations, the occurrence of the allelic variants 57L+58R (29%) and 57L+58R+61M+117T (19%) were most common in PNG. Prevalence of infections with the mutation Y976F in *pvm-dr1* was also high (40%) and the mutation was found in all possible combinations with the different genotypes detected in *pvdhfr*.

Our observations are consistent with the results from similar studies done in different countries in South East Asia, Central and South America and other parts of Oceania, where *P. falciparum* and *P. vivax* are sympatric and increasing levels of CQ resistance have led to a policy change to the alternative low cost option of SP. In these regions, *in vivo* resistance to SP in *P. falciparum* developed rapidly after its initial deployment as monotherapy (Baird, 2004; Peters, 1998) and was paralleled by the development of *in vivo* resistance in *P. vivax* (Pukrittayakamee *et al.*, 2000; Tjitra *et al.*, 2002). Results which were further corroborated by

the more recent demonstration of a similar molecular mechanism of antifolate resistance in both species, one that is conferred by single point mutations in the target enzymes of antifolates (i.e., *pvdhfr* and *pvdhps*, respectively) and is driven by exertion of selective drug pressure and progresses rapidly (Imwong *et al.*, 2003; Korsinczky *et al.*, 2004; Leartsakulpanich *et al.*, 2002; de Pécoulas *et al.*, 1998a). Different epidemiological studies determining the molecular *pvdhfr* background in field isolates originating from various regions worldwide, such as Thailand (Brega *et al.*, 2004; Imwong *et al.*, 2001), Indonesia (Hastings *et al.*, 2004; Hastings *et al.*, 2005; Tjitra *et al.*, 2002), Cambodia (Eldin de Pécoulas *et al.*, 2004), Myanmar (Na *et al.*, 2005), India (Kaur *et al.*, 2006; Valecha *et al.*, 2006), and Ethiopia (Schunk *et al.*, 2006), have shown that previous SP use is correlated with the prevalence rates of resistant *pvdhfr* alleles. Moreover, the association between infecting *pvdhfr* alleles and treatment outcome with SP monotherapy could be demonstrated in Thailand (Imwong *et al.*, 2001) and Indonesia (Hastings *et al.*, 2004; Tjitra *et al.*, 2002).

A similar development of SP resistance in *P. vivax* seems to have taken place in PNG, though SP was introduced in combination with the 4-aminoquinolines AQ and CQ. The primary aim of combination therapy is the prevention of the development and spread of resistance and is dependent on the efficacy and pharmacokinetic properties of each partner drug (Kremsner & Krishna, 2002; White, 1999).

Low sensitivity to SP in both species has been documented previously in PNG (Darlow *et al.*, 1982b), most probably been arisen because of former drug pressure exerted by mass treatment campaigns with pyrimethamine (in combination with CQ) in the late 1960s and 1970s (Spencer, 1994) and SP use in combination with quinine as second-line regimen against treatment failure and severe malaria. Therefore, the high frequency of pyrimethamine-specific molecular markers we measured in the *P. vivax* population is not surprising. Reports about moderate mutation rates of resistance markers in *P. falciparum dhfr* (i.e., S108N, C59R; Reeder *et al.*, 1996), which had reached almost fixed levels in 2003 (Marfurt *et al.*, 2006, in preparation, Chapter 4), provides further evidence for the hypothesis, that moderately resistant *dhfr* alleles had already occurred before the effective implementation of SP as part of the standard first-line treatment, and that the rapid emergence of high-level resistant alleles could not be curbed by its combination with AQ or CQ. The similar molecular mechanisms underlying antifolate resistance resulted in a similar course of the development of resistance in both *Plasmodium* species in PNG. Mainly because treatment in PNG is given based on presumptive clinical malaria diagnosis and therefore, both species are simultaneously under selection pressure of the same antimalarial drugs.

Though *in vivo* studies are still the gold standard for the determination of antimalarial drug efficacy, the assessment of molecular resistance markers has become an important complementary method for the monitoring of drug resistant *P. falciparum* malaria. However, since clinical treatment outcome is dependent on several environmental, host and parasite factors, the usefulness of marker sets can vary between areas and have to be established and evaluated for a given area (Alifrangis *et al.*, 2003, Omar *et al.*, 2001, Staedke *et al.*, 2004). The supplementation of *in vivo* efficacy data with molecular correlates could also be a valuable tool in monitoring *P. vivax* resistance, particularly because unambiguous determination of treatment failure rates is aggravated by difficulties in distinguishing relapses and new infections. Recent advances in the understanding of the mechanisms underlying SP resistance in *P. vivax* have paved the way for the molecular monitoring of resistance against antifolates in this species. However, molecular resistance markers for 4-aminoquinolines have not been reported for *P. vivax* up to present. Though orthologous genes for *pfCRT* and *pfmdr1*, two important genes involved in CQ resistant falciparum malaria (reviewed in Bray *et al.*, 2005; Duraisingh & Cowman, 2005), have been discovered and non-synonymous point mutations have been described, an association between resistance and these SNPs or other genetic alterations, such as gene amplification or varying expression levels, could not be established until now (Brega *et al.*, 2005; Nomura *et al.*, 2001; Sà *et al.*, 2005). Moreover, the elucidation of an association between molecular correlates and treatment outcome with combination regimens containing two drug classes being effective against different parasite targets may be more complex.

In order to suggest useful markers for the molecular monitoring of *P. vivax* resistance to AQ or CQ plus SP, we did a baseline assessment of the molecular profile in *P. vivax dhfr* and *mdr1* and investigated the association between infecting *pvdhfr/pvmdr1* genotypes and *in vivo* treatment response. Regarding *pvdhfr*, single point mutations 57L or 57I, 58R, 61M, and 117T, as well as the total number of mutations were all independently associated with an increased risk of treatment failure. These results are in concordance with previous results showing that 1) parasite reduction ratio 48 hours after initiation of treatment with SP was smaller in patients harbouring triple *dhfr* mutants than those harbouring double mutants (Imwong *et al.*, 2001), and 2) people infected with quadruple *dhfr* mutants were at higher risk to fail treatment with SP (Hastings *et al.*, 2004; Tjitra *et al.*, 2002). Regarding the combinations of mutations in *pvdhfr*, the observations that 1) mutation 117N was never observed in *dhfr* triple or quadruple mutants, 2) triple and quadruple mutants had always the mutation 117T, 3) 57L/I was always linked to 58R, and 4) 61M was only seen in quadruple

mutants, were all in agreement with previous data. The most prevalent *dhfr* genotypes described in previous studies included single 117, double 58+117, triple 117+58+57, and quadruple 117+61+58+57 mutants and because of this frequently observed allele structure, the stepwise accumulation of mutations in *pvdhfr* was suggested to be similar to that in *P. falciparum*, where low level pyrimethamine resistance is conferred by the single *pfdhfr* mutation 108N (corresponding to *pvdhfr* 117N) and drug selection processes leading to the addition of 59R and/or 51I (corresponding to *pvdhfr* 58R and 57L/I) increase resistance to SP (Imwong *et al.*, 2001; Sibley *et al.*, 2001; Tjitra *et al.*, 2002). Our data from PNG showing a high frequency of infections having the double mutant 57L+58R seem to be inconsistent with this hypothesis. Nevertheless, the same double mutant has been previously described in Thai and Indian field isolates (Imwong *et al.*, 2001; Kaur *et al.*, 2006) and Hastings *et al.* (2005) have reported prevalence rates of 5.3% in the Wosera area in 1999 and 8% in the North Coast area in 2000, respectively. Moreover, by using a yeast expression system for the investigation of *in vitro* drug sensitivity of different allelic *pvdhfr* variants, the same authors showed that the double mutant 57L+58R was less sensitive to pyrimethamine by a factor of seven when compared to the population expressing the wild-type allele.

Regarding *pvmr1*, we could confirm previous results that demonstrated the presence of the polymorphic *mdr1* locus Y976F in field isolates (Brega *et al.*, 2005). Furthermore, we found the mutation to be a strong independent predictor of treatment failure with AQ or CQ plus SP. To our knowledge, these are the first data that indicate *pvmr1* to play an important role in mediating drug resistance in *P. vivax*. This is in contrast with recent studies, where an association of *pvmr1* polymorphisms and *P. vivax* resistance to CQ and mefloquine could not be shown (Sà *et al.*, 2005; Picot *et al.*, 2005), the most likely reason being that these studies used very small sample sizes and were not specifically designed to demonstrate an association between *pvmr1* polymorphisms and *in vivo* treatment response. However, the role of *pvmr1* in conferring resistance to different drugs still remains to be clarified. On one hand, the situation may be equally complex as in *falciparum* malaria, where different SNPs and/or gene amplification were shown to be associated with resistance to 4-aminoquinolines, amino-alcohols and artemisinin derivatives, respectively (Duraisingh & Cowman, 2005; Duraisingh & Refour, 2005). On the other hand, in consideration of the differences in biology, pathophysiology and evolutionary history between these two *Plasmodium* species, the principal function of *pvmr1* and its contribution to a drug resistant phenotype may be completely different.



The good predictive value of the single markers analysed in both genes were further confirmed by the investigation of the relationship of the combined *pvdhfr/pvmdr1* genotype and treatment response, where the highest risk of failure was found to be significantly associated with an infecting genotype having a quadruple mutation in *pvdhfr* plus 976F mutation in *pvmdr1*. The observation of treatment failures with infections harbouring wild-type alleles may well be a consequence of technical constraints due to the limitations of the present technology to differentiate true recrudescences from relapses and/or new infections, which may have lead to an overestimation of true failure rates.

Though more elaborate, the application of sequencing methods would have been more informative, since the high plasticity of the *P. vivax* genome is known (Feng *et al.*, 2003; Imwong *et al.*, 2006) and a high diversity in *pvdhfr* alleles has already been reported from different geographic areas (Hastings *et al.*, 2004; Imwong *et al.*, 2003; Kaur *et al.*, 2006; Tjitra *et al.*, 2002).

In spite of these limitations and the fact that a drug resistant *P. vivax* phenotype is most likely mediated by multigenic processes, we think that the set of SNPs included in our study is sufficient to monitor parasite resistance under the current first-line regimen. The difference in treatment failure rates between sites was not only reflected in different prevalence rates of key markers which have shown an association with treatment response, but was also reflected in different frequencies of highly mutated and/or wild-type *pvdhfr/pvmdr1* genotypes circulating at the respective sites. These findings strongly support the usefulness of molecular markers to monitor the dynamics of *P. vivax* resistance and thus their important role in complementing *in vivo* efficacy data to decide on the most appropriate and feasible drug policy against vivax malaria. For the time being, we propose to use polymorphisms in *pvdhfr* F57L/I, T61M, and S117T/N plus *pvmdr1* Y976F for the molecular assessment of *P. vivax* resistance against AQ or CQ plus SP in PNG. However, including other SP relevant markers (e.g. polymorphisms in *pvdhps*), and as yet unidentified markers involved in resistance to other antimalarials, may become necessary for the longitudinal monitoring of resistance in the future, in particular when a policy change will recommend new classes of drugs.

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**TABLE 1:** Sequences of primers and oligonucleotide probes used for the detection of *pvdhfr* (F57L/I, S58R, T61M, S117T/N, and I173F/L) and *pvmdr1* (Y976F and F1076L) mutations

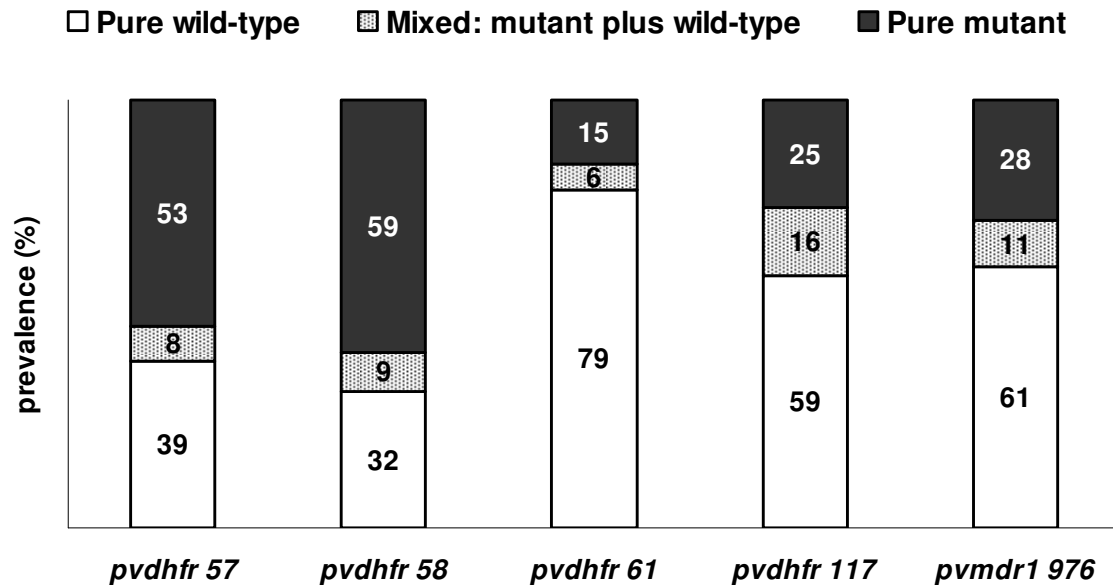
Gene	Oligonucleotides	Sequence (5' - 3')	Position
<i>pvdhfr</i>		<sup>a</sup> Accession number: X98123 (PCR product: 422 bp)	
	<i>pvdhfr</i> S	5'-TCTGGGCAATAAGGGGACT-3'	114 - 132
	<i>pvdhfr</i> A	5'-AGTTTCTACTTAGGCATTCCCTAT-3'	559 - 536
	Sensor 57/8	5'-GTAGGTCGTCACCGAGCTGAAGT FL-3'	189 - 167
	Anchor 57/8	5'-CTTCATATCGACGGAGTTGCATTTCCATG PH-3'	165 - 137
	Sensor [G]	5'-GATGCTCTCCCAGCTGCTTC FL-3'	363 - 344
	Anchor 117	5'-CCCCATGACCACGACGTTTTTGCAG PH-3'	342 - 319
	Sensor 172V	5'-TGTGCTCCCCCAATGACGA FL-3'	530 - 512
	Anchor 172/173	5'-GCATTTGTAGTACTTCAGCTTCTTTAAGAGC PH-3'	510 - 480
<i>pvmdr1</i>		<sup>a</sup> Accession number: AY618622 (PCR product: 763 bp)	
	<i>pvmdr1</i>	5'-ATAGTCATGCCCCAGGATTG-3'	2753-2772
	<i>pvmdr1</i> 447AS	5'-ACCGTTTGGTCTGGACAAGTAT-3'	3535-3516
	Sensor Phe	5'-CATAAAAATGAAGAACGTTCCGGTC FL-3'	2940-2916
	Anchor 976	5'-GTACAGCCGCCACGATAGGGCAGAA PH-3'	2914-2890
	Sensor Leu	5'-AGTGCCCAACTTTTCATTAACAG FL -3'	3217-3239
	Anchor 1076	5'-TTGCCTACTGGTTTGGTTCCTTCCT PH-3'	3242-3266

*Pvdhfr*, *P. vivax* dihydrofolate reductase; *pvmdr1*, *P. vivax* multidrug resistance gene 1; <sup>a</sup> GenBank™ database (<http://www.ncbi.nlm.nih.gov>) accession number; PCR, polymerase chain reaction; bp, base pairs



**TABLE 2:** Baseline characteristics of patients at enrolment and treatment outcomes for amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against *P. vivax* malaria

Study site	North Coast area (Madang Province)	Karimui area (Simbu Province)	South Wosera area (East Sepik Province)	Total
Characteristics	n=34	n=43	n=27	n=104
Weight (mean (95% CI), kg)	15.9 (8.0-23.8)	13.7 (12.2-15.2)	12.0 (10.6-13.5)	14.1 (11.2-16.9)
Age (mean (95% CI), yrs)	2.3 (1.9-2.7)	3.5 (3.0-4.0)	3.2 (2.5-3.9)	3.0 (2.7-3.4)
Sex: females/n (%)	20 (58.8)	17 (39.5)	9 (36.0)	47 (45.2)
Temperature (mean (95% CI), °C)	37.1 (36.6-37.6)	38.6 (38.3-38.8)	37.0 (36.4-37.6)	37.7 (37.4-38.0)
Haemoglobin (mean (95% CI), g/dl)	10.2 (9.4-11.0)	10.6 (10.0-11.2)	9.2 (8.6-9.8)	10.1 (9.7-10.5)
Parasite density (geometric mean (range), per µl)	4677 (300-41280)	3437 (40-36600)	4964 (160-50640)	4182 (40-50640)
Class	no (%)			
Adequate clinical and parasitological response (ACPR)	24 (70.6)	40 (93.0)	27 (100)	91 (87.5)
Treatment failure (TF)	<b>10 (29.4)</b>	<b>3 (7.0)</b>	<b>0 (0)</b>	<b>13 (12.5)</b>



**FIGURE 1:** Prevalence of mutations in *pvdhfr* (*P. vivax* dihydrofolate reductase) and *pvmdr1* (*P. vivax* multidrug resistance gene 1) assessed in pre-treatment samples from patients with a *P. vivax* mono-infection in Papua New Guinea. Each patient sample was determined as either pure mutant allele, or pure wild-type allele, or mixed allele infection, respectively.

**TABLE 3:** *Pvdhfr* haplotypes in pre-treatment samples from patients in Papua New Guinea who received amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against *P. vivax* infection

<i>Pvdhfr</i> polymorphism				No of samples
57	58	61	117	
<b>Samples with single <i>pvdhfr</i> haplotype</b>				<b>78</b>
F	S	T	S	28
F	S	T	N	3
F	<b>R</b>	T	S	1
F	<b>R</b>	T	<b>T</b>	2
F	<b>R</b>	T	N	3
<b>L</b>	<b>R</b>	T	S	25
<b>L</b>	<b>R</b>	T	<b>T</b>	1
<b>L</b>	<b>R</b>	<b>M</b>	<b>T</b>	14
<b>I</b>	<b>R</b>	<b>M</b>	<b>T</b>	1
<b>Samples with mixed <i>pvdhfr</i> haplotypes</b>				<b>22</b>
F	<b>S/R</b>	T	S	1
F	S	T	S/T	1
<b>L</b>	<b>R</b>	T	S/T	4
<b>L</b>	<b>R</b>	T	S/N	5
<b>L</b>	<b>R</b>	T/M	S/T	2
<b>L</b>	<b>R</b>	T/M	<b>T</b>	1
F/L	<b>S/R</b>	T	S	4
F/L	<b>S/R</b>	T	S/N	1
F/L	<b>S/R</b>	T/M	S/N	1
F/L	<b>S/R</b>	T/M	S/T	2
Total number of samples				<b>100</b>

*Pvdhfr*, *P. vivax* dihydrofolate reductase; bold, mutated alleles

**TABLE 4:** Association between single mutated gene loci in *pvdhfr* and *pvmdr1* and treatment response to amodiaquine or chloroquine plus sulphadoxine-pyrimethamine

Polymorphic SNP <sup>a</sup> sites in <i>pvdhfr</i> and <i>pvmdr1</i>	P(%) <sup>b</sup>	Treatment outcome		OR <sup>e</sup>	CI <sup>f</sup>	p <sup>g</sup>
		ACPR <sup>c</sup>	TF <sup>d</sup>			
<i>pvdhfr</i> Mut57 <sup>h</sup>	61	49	12	9.31	1.16-74.75	<b>0.01</b>
<i>pvdhfr</i> 57L	60	49	11	4.27	0.89-20.40	0.05
<i>pvdhfr</i> 57I	1	0	1	*		<b>0.01</b>
<i>pvdhfr</i> 58R	68	56	12	6.64	0.82-53.52	0.04
<i>pvdhfr</i> 61M	21	15	6	4.11	1.21-13.99	0.02
<i>pvdhfr</i> Mut117 <sup>i</sup>	41	33	8	2.62	0.79-8.68	0.11
<i>pvdhfr</i> 117T	28	20	8	5.36	1.58-18.23	<b>0.01</b>
<i>pvdhfr</i> 117N	13	13	0	§		0.14
<i>pvmdr1</i> 976F <sup>k</sup>	39	30	9	4.28	1.22-15.04	<b>0.02</b>
<i>pvmdr1</i> 976Y <sup>l</sup>	72	67	5	0.18	0.04-0.74	<b>&lt;0.01</b>

<sup>a</sup> SNP, single nucleotide polymorphism; *pvdhfr*, *P. vivax* dihydrofolate reductase; *pvmdr1*, *P. vivax* multidrug resistance gene 1; <sup>b</sup> P, prevalence; <sup>c</sup> ACPR, adequate clinical and parasitological response; <sup>d</sup> TF, treatment failure; <sup>e</sup> OR, odds ratio; <sup>f</sup> CI, 95% confidence interval; <sup>g</sup> calculated by standard  $\chi^2$  analysis or Fisher's exact test; <sup>h</sup> Mut57, either 57L or 57I; <sup>i</sup> Mut117, either 117T or 117N; <sup>k</sup> 976F represents mutated allele; <sup>l</sup> 976Y represents wild-type allele; \* 57I polymorphism was found in only one patient who failed treatment; § 117N was not found in patients who failed treatment

**TABLE 5:** Association between infecting *pvdhfr/pvmdr1* genotypes and treatment response to amodiaquine or chloroquine plus sulphadoxine-pyrimethamine

<i>pvdhfr/pvmdr1</i> genotypes <sup>a</sup>		No (x)	Treatment response		OR <sup>d</sup>	CI <sup>e</sup>	<i>p</i> (LRT <sup>f</sup> )
No of <i>pvdhfr</i> mutations	<i>pvmdr1</i> polymorphism		ACPR <sup>b</sup>	TF <sup>c</sup>			
Wild-type	976Y <sup>g</sup>	19	18	1	0.32	0.04-2.62	0.22
Wild-type	976F <sup>h</sup>	9	9	0			
Single 117	976Y	3	3	0			
Single 117	976F	1	1	0			
Single 58	976Y	1	1	0			
Single 58	976F	1	1	0			
Double 57-58	976Y	18	17	1	0.34	0.04-2.82	0.26
Double 57-58	976F	11	8	3	2.96	0.67-13.03	0.18
Double 58-117	976Y	4	4	0			
Double 58-117	976F	1	1	0			
Triple 57-58-117	976Y	5	4	1	1.73	0.18-16.79	0.65
Triple 57-58-117	976F	6	5	1	1.37	0.15-12.72	0.79
Quadruple 57-58-61-117	976Y	11	10	1	0.64	0.08-5.47	0.67
Quadruple 57-58-61-117	976F	10	5	5	<b>10.25</b>	<b>2.44-43.11</b>	<b>&lt;0.01</b>
Total (n)		100	87	13			

*Pvdhfr*, *P. vivax* dihydrofolate reductase; *pvmdr1*, *P. vivax* multidrug resistance gene 1; <sup>a</sup> the genotype is assigned according to the mutated alleles (i.e., mixed allele is coded as mutant); <sup>b</sup> ACPR, adequate clinical and parasitological response; <sup>c</sup> TF, treatment failure; <sup>d</sup> OR, odds ratio; <sup>e</sup> CI, 95% confidence interval; <sup>f</sup> LRT, likelihood ratio test; <sup>g</sup> wild-type allele; <sup>h</sup> mutated allele

**TABLE 6:** Prevalence of polymorphisms in *pvdhfr* and *pvm-dr1* and corresponding treatment failure rates with amodiaquine or chloroquine plus sulphadoxine-pyrimethamine at three different sites in Papua New Guinea

Study site	Prevalence (%)			<i>P</i> ( $\chi^2_{(2)}$ )
	North Coast area	Karimui area	South Wosera area	
<b>Mutated SNP<sup>a</sup> sites</b>	n=30	n=43	n=27	
<i>pvdhfr</i> Mut57 <sup>b</sup>	83.33	48.84	55.56	<b>0.01</b>
<i>pvdhfr</i> 57L	83.33	46.51	55.56	<b>0.01</b>
<i>pvdhfr</i> 57I	0.00	2.33	0.00	0.51
<i>pvdhfr</i> 58R	90.00	55.81	62.96	<b>0.01</b>
<i>pvdhfr</i> 61M	26.67	11.63	29.63	0.13
<i>pvdhfr</i> Mut117 <sup>c</sup>	53.33	27.91	48.15	0.06
<i>pvdhfr</i> 117T	40.00	18.60	29.63	0.13
<i>pvdhfr</i> 117N	13.33	9.30	18.52	0.54
<i>pvm-dr1</i> 976F	70.00	25.58	25.93	<b>&lt;0.001</b>
<b>Wild-type SNP sites</b>				
<i>pvdhfr</i> 57F	30.00	55.81	51.85	0.08
<i>Pvdhfr</i> 58S	23.33	51.16	44.44	0.05
<i>pvdhfr</i> 61T	86.67	90.70	74.07	0.16
<i>pvdhfr</i> 117S	73.33	83.72	62.96	0.14
<i>pvm-dr1</i> 976Y	46.67	81.40	85.19	<b>0.001</b>
<b>TF<sup>d</sup> rate (x/n (%))</b>	<b>10 (33.3)</b>	<b>3 (7.0)</b>	<b>0 (0.0)</b>	<b>&lt;0.001</b>

<sup>a</sup> SNP, single nucleotide polymorphism; *pvdhfr*, *P. vivax* dihydrofolate reductase; *pvm-dr1*, *P. vivax* multidrug resistance gene 1; <sup>b</sup> Mut57, either 57L or 57I; <sup>c</sup> Mut117, either 117T or 117N;

<sup>d</sup> TF, treatment failure

## **Chapter 8**

### **Discussion**

Accurate diagnosis and effective treatment of malaria is still the mainstay in the control of the disease. However, the emergence and spread of parasite resistance to the commonly used drugs (i.e., 4-aminoquinolines and antifolates) has aggravated the burden of malaria during the last decades (Björkman & Bhattarai, 2005). The problem is complex, but several measures can be taken to restrain the scale and impact of parasite resistance:

- 1) Adopt adequate methods to assess the level of parasite resistance
- 2) Protect current drugs against resistance by using combination therapy
- 3) Expand access to prompt and effective treatment for those in most need
- 4) Promote evidence-based drug policies and sensible practices
- 5) Encourage and sustain efforts for the development of new antimalarial compounds (Olliaro, 2005).

The current studies gave clear consideration to point one: They investigated current and novel *in vivo* and molecular approaches in assessing parasite resistance of falciparum and vivax malaria in Papua New Guinea.

This chapter will first give a general overview of common issues encountered with antimalarial drug policy in the face of drug resistance. The following paragraphs will then summarise again the main findings of the current studies, which are discussed in detail in the corresponding chapters 2 to 7, highlight their main implications for Papua New Guinea (PNG), and also discuss their relevance in the broader context of drug resistant malaria surveillance. The chapter will place emphasis on the main issues associated with *in vivo* and molecular assessment of parasite resistance, with a special focus on the feasibility and applicability of molecular monitoring approaches and their potential role in helping to monitor resistance and make decisions on rational treatment strategies against malaria.

### **1. Evidence-based antimalarial drug policy**

Effective antimalarial drug policy should be characterised by clear formulation of objectives and targets, effective implementation and regular up-dates, and requires several components to be in place. These include:



- Regulations to support and enable the policy
- Adequate drug supply
- Guidelines on the use of antimalarial drugs
- Training on diagnosis and treatment of malaria
- Standardised mechanisms to regularly reassess the safety, efficacy and effectiveness of the implemented policy (Bloland & Ettling, 1999).

Drug efficacy is commonly regarded as the most important determinant for effectiveness. However, if operational factors are not adequate, even a very efficacious drug regimen will not lead to good effectiveness on national level. Therefore, the evaluation of operational factors, as well as behavioural factors in the target population where policy has to be implemented, is important to be considered in the decision-making process. These include important aspects, such as

- The quality of drugs
- Adequate drug supply on central and peripheral levels
- Appropriate health care provision by public and private sector
- Access to health care facilities
- Treatment-seeking behaviour in the communities
- Acceptance of and compliance to treatment.

Therefore, alongside the assessment of clinical efficacy in controlled studies, the measurement of process indicators of successful implementation of antimalarial drug policy are important additional data in order to estimate the likely effectiveness of the policy (Amin *et al.*, 2004; Fevre & Barnish, 1999).

Revision of antimalarial policy in previous years was mainly dependent on the country's resources and its public-health infrastructure. Decisions to change treatment policy, especially in Sub-Saharan Africa, were mainly based on economical reasons (Amin *et al.*, 2004; Fevre & Barnish, 1999; Schellenberg *et al.*, 2006). The most important impediments to policy development include the lack of consensus on the methods to collect, analyse and present drug efficacy data, insufficient information on mechanisms of resistance and consequently,

the lack of consensus on tolerable levels of resistance. Hence, there is a strong need for evidence-based assessments in order to maximise the impact of the current policy and to decide on when and how a policy change should be initiated (Shretta *et al.*, 2000).

Finally, other measures and/or interventions of national malaria control programs which limit the development and spread of resistance include:

- Treatment based on definitive malaria diagnosis
- Measures to increase compliance (i.e., supervision of treatment, pre-packaged treatment doses)
- Effective re-treatment of parasitological failures to prevent progress to clinical disease
- Combination therapy with two different drug classes
- Strategies to reduce transmission intensity by preventing human-to-vector contact (i.e., vector control measures, insecticide-treated bednet programs).

Though the above mentioned malaria control activities may well benefit the effectiveness of treatment policy, special attention has to be given to more recently promoted drug-based intervention strategies, such as intermittent preventive treatment (IPT) for infants and/or pregnant women. Careful monitoring of drug-based interventions is critical in order to predict their potential impact on current and possible future antimalarial treatment strategies (Schellenberg *et al.*, 2006).

### **1.1 Indicators for policy change**

Only a few publications are available on the issue of antimalarial drug policy in the face of drug resistance and decision criteria on when and how drug policy should be changed are still controversial (Bloland & Ettling, 1999; Fevre & Barnish, 1999; Schapira *et al.*, 1993; WHO, 2005). It is apparent that there are several aspects to consider when making decisions on national drug policies. Among the many different factors, which include costs, availability and acceptability of drugs, prescribing practices of public- and private-sector facilities, patient and provider compliance with drug policies, and treatment-seeking behaviour of the communities, efficacy of both, current and proposed alternative treatment regimens, is of major importance (Bloland & Ettling, 1999). Since the principal objective of antimalarial treatment is the reduction of malaria morbidity, and especially mortality, it seems reasonable

to use morbidity and mortality rates as indicators to decide on whether a national treatment strategy is satisfactory. Unfortunately, a high proportion of the disease burden is not captured by data collected by routine surveillance at health facilities. Consequently, reliable data on drug efficacy are not available outside clinical and/or epidemiological studies (Winstanley *et al.*, 2002).

At the present time, *in vivo* drug efficacy studies are still the ‘gold standard’ method for the assessment of drug resistant malaria and *in vivo* drug efficacy is still accepted as one of the most important indicators for the initiation of a policy change. However, common indicators, which are applicable in different epidemiological settings and for various drugs, are difficult to define. Several criteria, based on outcome measures of *in vivo* drug efficacy studies have been suggested, such as the level of parasite resistance, i.e., RIII resistance between 5 and 30% (Bloland *et al.*, 1993; Sudre *et al.*, 1992), or the level of *in vivo* failure rate >15-20%. Definitions based on *in vivo* failure rates were as yet primarily based on outcomes using the standardised Day 14 follow-up protocol (WHO, 1996). In order to provide evidence and guidance for required actions in the decision-making process, a systematic approach was suggested by Andrew Kitua (2000). The process was divided into four main periods which included:

- 1) The *grace* period (clinical failure rate <5%), in which there is time for epidemiological, health systems, drug dynamics, and behavioural research,
- 2) The *alert* period (clinical failure rate <15%), in which clear and systematic actions can be developed and planned,
- 3) The *action* period, sub-divided into:
  - i) Early *action* period (clinical failure rate 15-20%), and
  - ii) Accelerated *action* period (clinical failure rate 21-24%), in which specific actions, such as the ascertainment of treatment failure rates and the evaluation of alternative treatment regimens (i.e., assessment of safety and efficacy profile and costs) are performed, and
- 4) The *change* period (clinical failure rate  $\geq$ 25%), where consensus based on relevant actions has been reached and respective policy change has to be implemented.

However, this system was based on outcome measures with the WHO standard protocol from 1996, which was drawn up primarily for high transmission areas. The protocol has been

challenged by several authors (Plowe *et al.*, 2001; White, 2002) and has thereupon been recognized not to be sensible for low-to-moderate transmission areas (Ruebush *et al.*, 2003; WHO, 2003).

Recently published recommendations from WHO suggest that clinical, as well as parasitological resistance should be considered. The reason being that parasitological failure rates are likely to translate into clinical failure rates, either within a short-term in the infected individual depending on the immunological status, or within a long-term on population level as parasite resistance increases (Mutabingwa *et al.*, 2001; Njama-Meya *et al.*, 2004; WHO, 2006). Moreover, asymptomatic parasitaemia after treatment is associated with an increased risk of anaemia, gametocyte carriage, and gametocyte-infectivity for mosquitoes (Hogh *et al.*, 1998; Price *et al.*, 2001; Sowunmi *et al.*, 2004). Therefore, the standard efficacy test protocol has been revised recently (WHO, 2001a; WHO, 2003) and can be applied for low-to-moderate and high transmission areas. Furthermore, the revised protocol uses a common classification scheme for all transmission areas and suggests duration of follow-up to be 28 days for drugs with elimination half-lives <7 days (AQ, CQ, QUIN, HAL, SP, and Malarone<sup>TM</sup>), and 42 days for MEF and LUM (Stepniewska *et al.*, 2004). The new WHO protocol takes into account both, clinical and parasitological results, and strongly suggests applying molecular techniques for the differentiation of recrudescences from new infections. However, different studies usually apply protocols that are based on different genotyping markers (i.e., polymorphic genes, such as *msp1*, *msp2* and *glurp*, or microsatellite and/or SNP analysis). Since classification of genotyping results can have profound influence on the assessment of drug efficacy (Slater *et al.*, 2005), there is a need for both, definitions for sensitivity of detection of molecular methods, as well as the classification and analysis of the resulting genotyping data.

The current WHO guidelines recommend that a policy change should be considered when total failure rate is  $\geq 25\%$  and clinical failure rate is  $\geq 15\%$ . With combination regimens, a change is indicated at a level of a total failure rate at Day 28 of  $\geq 10\%$  (WHO 2005; WHO, 2006).

## 1.2 The role of molecular markers

Systematic studies have shown that decreasing *in vitro* sensitivity in local field isolates can give an early indication for raising *in vivo* resistance (Brockman *et al.*, 2000; Huong *et al.*, 2001). Similarly, where molecular correlates for resistance are known, the assessment of the prevalence of mutated alleles can serve as an early warning tool for the development of

resistance (Djimde *et al.*, 2001a; Kublin *et al.*, 2002). However, *in vitro* sensitivity tests and the assessment of molecular markers are still considered research tools.

Molecular markers are recommended as complementary tool to the current ‘gold standard’ of *in vivo* drug efficacy tests. But because the number of laboratories with the required infrastructure and expertise is still small in developing countries, molecular monitoring is recommended to be restricted to reference centres at sentinel sites. Molecular methods have been successfully applied in various epidemiological settings, including countries with limited resources, and have produced a vast amount of valuable data. However, the assessment of molecular markers as a routine monitoring tool is still complicated due to several questions and issues which call for resolution and improvement:

- 1) There is a lack of standardised guidelines for an appropriate design for molecular studies that can be used in different epidemiological settings and for different drug regimens.
- 2) There is a need for molecular techniques that are easy to use, cheap, and allow high throughput of samples. In addition, standard operating procedures for blood sampling, DNA extraction, and further downstream molecular analyses are required.
- 3) Molecular data do not relate directly to clinical treatment outcome. Apart from many host and environmental factors which determine *in vivo* treatment response, to what extent does parasite resistance play a role?
- 4) Once the relationship of molecular correlates and *in vivo* treatment response is established for several drugs and combinations, how are these results translated into standardised parameters and/or indices for parasite resistance?
- 5) Once these parameters and/or indices for parasite resistance are found, how relevant will they become for the decision-making process on antimalarial drug policies?

## **2. *In vivo* assessment of antimalarial drug efficacy**

As in many malaria endemic areas in Africa, Asia, and South America, the development and spread of resistance to the commonly used antimalarial drugs represents a challenge for the control of the disease in Papua New Guinea (PNG). In response to the occurrence of widespread resistance to 4-aminoquinoline drugs, PNG decided to replace 4-aminoquinoline monotherapy with AQ or CQ plus SP in 1997. This decision against an artemisinin-based combination therapy (ACT) was not only taken because of economical reasons. The second

dominant *Plasmodium* species in PNG is *P. vivax* and malaria diagnosis at health facilities is usually based on clinical criteria. Therefore, effectiveness against both species was an important argument in favour of this combination because a) it was thought that SP, when used as monotherapy, would have reduced efficacy against *P. vivax* malaria, and b) 4-aminoquinolines were still efficacious against non-falciparum malaria. When first efficacy trials with the new combination regimen conducted between 1998 and 1999 had shown good efficacy (treatment failure rates below 5%) against *P. falciparum* malaria (Jayatilaka *et al.*, 2003), the PNG Department of Health has replaced the standard first-line therapy with AQ or CQ against uncomplicated malaria with the combination of AQ or CQ plus SP in the year 2000.

In our *in vivo* efficacy studies conducted between 2003 and 2005 at three different sites in PNG we measured PCR-corrected treatment failure rates for *P. falciparum* malaria up to Day 28 between 12% and 28%, depending on the region and the year of assessment. Overall treatment failure rate in *P. vivax* malaria was 12%. Unfortunately, comparison with previous data was hampered since former studies were using follow-up periods of 14 days and genotyping methods for the distinction between recrudescences and new infections were not applied. By restricting the analysis in our studies to the Day 14 outcomes based on clinical and parasitological criteria alone, we measured treatment failure rates between 2% and 18%. These data implied that there was a two to threefold decrease in efficacy of AQ or CQ plus SP only three years after effective implementation of the new first-line regimen in PNG. Though clinical failure rates were still low (<10% at all three sites), overall treatment failure rates exceeded 12% in all three sites.

Our data clearly highlight recently raised controversial issues associated with the assessment of antimalarial drug efficacy (White, 2002) which are discussed in the following paragraphs.

In concordance with previous data, our results demonstrate that *in vivo* studies with a follow-up period of 14 days are not sensitive enough to assess the therapeutic efficacy of the current first-line regimen in the country (Mugittu *et al.*, 2005; Stepniewska *et al.*, 2004). Assessment up to Day 14 clearly underestimated the true failure rate. In our studies, in both, low-to-moderate and high transmission areas, the majority of patients had recurrent parasitaemia after Day 14. Furthermore, late recurrences (i.e., appearing after Day 14) had to be expected for the current regimen including drugs with long elimination half-lives, such as SP (White, 2002).

Our *in vivo* efficacy data, which were further corroborated by molecular data showing a highly CQ resistant and moderately SP resistant genetic profile in the parasite populations

(Chapters 4 to 7), indicate a relatively rapid loss of efficacy of the current first-line regimen in PNG and strongly argue for careful *in vivo* monitoring of drug efficacy in the country. The application of the newly revised WHO standard protocol (WHO, 2003) using a Day 28 follow-up is essential to prevent potential underestimation of failure rates. Furthermore, the use of molecular genotyping methods for the determination whether treatment failure samples harbour recurrent or new infections, is inevitable to prevent potential overestimation of failure rates.

Though the indication for genotyping methods is not open to debate, subsequent interpretation of results and data analysis can have significant effect on the outcome of a study. According to the newly revised WHO protocol (2003), cases of new infections with *P. falciparum*, as well as infections with *P. vivax* during the follow-up period, should be excluded from the study. The main reason being that rescue treatment given for a new infection could potentially mask a true recrudescence which was, in contrast to the new infection, not yet detectable by microscopy and PCR. The main problem with this recommendation is that the indication for rescue treatment is different for low-to-moderate and high transmission areas (i.e., rescue treatment is given to both, clinical and parasitological failures in the former areas, but to parasitological failures not until the end of follow-up in the latter areas, respectively). This may be the reason why analysis of data is not done consistently in different studies. In addition, a recent report from Uganda, that investigated the effect of different classification schemes for genotyping data on the estimates of treatment failure rates, clearly shows the need for a standardised protocol (Slater *et al.*, 2005). In our studies, we presented both, data unadjusted for genotyping, as well as PCR-corrected treatment failure rates. Adjustment for genotyping was done by classifying mixed genotypes (i.e., recrudescence plus new infection present) as treatment failures. Furthermore, cases whose recurrent parasites were identified as originating from a new infection, regardless whether mixed or pure, were not excluded from analysis. The main reason being that we used the current WHO recommendations for rescue treatment for high transmission areas at all three sites (i.e., patients were closely monitored up to Day 28 and rescue treatment was given at the end of the follow-up period). A similar approach (i.e., Kaplan-Meier analysis in conjunction with classifying new infections as adequate treatment responses) was also favoured recently by Guthmann and colleagues (2006), a decision which was based on the principle that as little information as possible should be discarded.

In order to adapt a standardised protocol for all three study sites, which represent different settings with regard to transmission intensity, that is still compatible with local conventions

and health care practices and logistically feasible, a minor modification to the WHO standard protocol was made. In order to reach the required sample size (i.e., 120 subjects per study) within a reasonable time frame, we included children between 6 months and 7 years of age. Though older children are more likely to have partial immunity, we could not find an association between either age and treatment outcome, nor the treatment regimen (i.e., SP plus AQ or CQ), which partially depends on age (i.e., AQ is given to children <14 kg under current first-line policy in PNG), and treatment outcome (Chapter 4). Though the issue has been addressed by age-stratified analysis of data, which did not show a difference in treatment failure rate between the two treatment categories (i.e., SP plus AQ or CQ), one might still speculate that CQ is less efficacious than AQ, since the proportion of children which were able to clear CQ resistant parasites might have been larger in the CQ plus SP-group (i.e., on average older children). However, it was the principle aim of our *in vivo* studies to determine treatment failure rates under the current first-line policy in PNG, and not to compare clinical efficacy of AQ plus SP versus CQ plus SP.

In view of the present situation in PNG, close monitoring is required of both, the current first-line (AQ or CQ plus SP), as well as the second-line (Artesunate for 7 days plus SP on Day 3) regimen. Furthermore, careful evaluation of potential candidate regimens for replacement is urgently needed, so that appropriate measures with regard to future policy change can be taken in due time. Combination regimens currently recommended by WHO include:

- Amodiaquine plus sulphadoxine-pyrimethamine,
- Artesunate plus sulphadoxine-pyrimethamine,
- Artesunate plus amodiaquine,
- Artemeter-lumefantrine (Coartem<sup>®</sup>), or
- Artesunate plus mefloquine (WHO, 2001b; WHO, 2006).

There is strong advocacy for artemisinin-based combination therapy (ACT) because artemisinins have the added advantage of diminishing gametocyte carriage and lowering infectivity to mosquitoes, at least in low endemic countries (Hallett *et al.*, 2004; Price *et al.*, 1996).

Considering the facts, that 1) evidence for the success of the combination regimen of AQ or CQ plus SP was scarce at that time (McIntosh & Greenwood, 1998), and 2) the success of a combination regimen is dependent on the efficacy of each partner drug (Kremsner & Krishna,



2002), and 3) high levels of 4-aminoquinoline resistance and reduced sensitivity to antifolates were reported in PNG (Chapter 2), limited efficacy of AQ or CQ plus SP had to be expected. However, non-artemisinin-based combination therapy (NACT) with AQ plus SP has recently been shown to be equally or more efficacious than ACT with SP in northern Ghana (Mockenhaupt *et al.*, 2005a) and Uganda (Sendagire *et al.*, 2005; Yeka *et al.*, 2005), the main reason being that resistance levels to SP and AQ were still low in these regions. Therefore, in certain regions where previous AQ and SP use was low, NACTs can still be considered as cost-effective *interim* options before full implementation of ACTs (Obonyo *et al.*, 2006), though the cost-effectiveness of such an *interim* option versus direct implementation of ACT has to be carefully evaluated (Laxminarayan, 2004). In contrast, previous drug history in PNG was clearly different (i.e., constant AQ pressure for more than 20 years and sporadic use of SP) and the prospect for the combination of AQ plus SP to work was therefore low.

ACTs have various advantages and disadvantages with respect to safety, tolerability, and efficacy, as well as risk of development of resistance, practicability and costs. The decision as to which treatment regimen to choose in PNG is therefore likely to be complex. Sound, locally acquired data will be necessary for this decision to be made in a sensible manner. But given the current *in vivo* and molecular resistance data in this report, this process becomes of utmost importance.

Many of possible replacement regimens may be difficult to implement in PNG due to cost (e.g. mefloquine), pre-existing resistance (AQ, CQ, and SP), or practicability (e.g., Coartem<sup>®</sup>).

Combination therapy with artesunate plus mefloquine has been successful against multi-drug resistant malaria in Thailand since 1995 (Wongsrichanalai *et al.*, 2001) and mefloquine alone has recently been shown to be effective against treatment failure malaria in PNG (Genton *et al.*, 2006), as well as against CQ resistant *P. vivax* malaria in Indonesian Papua (Maguire *et al.*, 2006). However, a mefloquine-based ACT option would have to be carefully evaluated with regard to the current health infrastructures in PNG, as well as large differences in malaria epidemiology within the country (Müller *et al.*, 2003; Wongsrichanalai *et al.*, 2000).

Other potential candidates include the combination of artesunate with the antifolate chlorproguanil plus dapsone (Lap-Dap<sup>TM</sup>-plus) or dihydroartemisinin-piperaquine (Artekin<sup>®</sup>). Though the latter combination has been shown to be well tolerated and efficacious in phase III trials (Tran *et al.*, 2004; Smithuis *et al.*, 2006), there are still gaps in the knowledge with regard to their suitability in PNG, in particular since data about cross-resistance between

piperaquine and AQ/CQ are not yet available. The triple combination Lap-Dap<sup>TM</sup>-plus is still under development, but similar concerns arise with regard cross-resistance between SP and the biguanide-dapsone combination Lap-Dap<sup>TM</sup>.

Final decisions on the exact choice of alternative drug regimens to be evaluated will most probably be based on availability and affordability and have to be made in consultation with PNG health policy makers. But as a matter of fact, in view of high-level resistance to AQ and CQ and rapidly progressing SP resistance, these drug classes should definitely not be considered as partner drugs in potential combination regimens in PNG. An artemisinin-based combination regimen should certainly be favoured and regarding the current status of antimalarial resistance in PNG, mefloquine seems to be one of the most promising partner drugs.

### **3. Molecular monitoring of parasite resistance**

As all life functions of the malaria parasite, drug resistance is genetically determined and advanced understanding of the molecular basis of drug resistance have provided novel tools to study this phenomenon (Greenwood, 2002). The identification of several genes encoding drug targets of the most commonly deployed antimalarials (i.e., aminoquinolines, antifolates, and artemisinin derivatives) and elucidation of genetic modifications conferring parasite resistance (i.e., primarily SNPs and in some instances, gene amplification) have led to the identification of several molecular markers for parasite resistance (described in detail in Chapters 4, 6 and 7) and molecular monitoring of parasite drug resistance has become a complementary tool for long-term surveillance and for developing predictive models on malaria drug resistance (Plowe, 2003).

#### **3.1 Assessment of molecular markers: technical aspects**

A prerequisite for the use of molecular markers in an epidemiological context, such as the surveillance of parasite resistance to antimalarial drugs, is the availability of rapid and reliable techniques for their identification. There is a need for methods that facilitate parallel analysis of multiple SNPs and are affordable and enable analysis of large sample sets. To date, a number of techniques have been developed and applied in different settings, including laboratories in resource-restricted countries. The majority is based on PCR-RFLP or sequencing analysis, and more recently developed techniques include real-time PCR and

MALDI-TOF analysis. However, since most of these techniques are limited by low sensitivity and/or specificity, high costs, and are not suitable to be applied on an epidemiological scale, we have developed a parallel SNP analysis system, which is based on PCR, primer extension and DNA microarray technology, and allows the determination of all known SNPs in drug resistance associated *P. falciparum* genes. In relation to previously used techniques, costs are significantly lower and large numbers of samples can be analysed in a reasonably short time (Chapter 3). Advantages and disadvantages of currently used methods and the new DNA microarray-based technique are summarized again in table 1 and ranked with regard to sensitivity, specificity, ability to detect multiple clone infections (MOI), throughput, handling, and costs.

Table 1: Comparison of different methods for SNP analysis

Technique	Sensitivity	Specificity	MOI	Throughput	Handling	Costs
PCR-RFLP	+++	+	+	+	intensive	++
Sequencing	++	+++	+/-	++	moderate	++
MALDI-TOF	+	+++	+/-	+++	moderate	+++
DNA microarray	+++	++/+++	++	++/+++	simple	+

The main advantage of the DNA microarray-based method is the possibility to analyse all known drug resistance associated SNPs at once. Hence, it might become possible to better elucidate the genetic background of drug failure, since phenotypic parasite resistance is likely to be mediated by multiple genes and as a consequence, conferred by SNPs from different loci. This underscores the need for linking individual SNPs into haplotypes. However, current algorithms and techniques are yet unable to generate true haplotypes of unlinked loci in samples containing multiple infections. The added advantage of the new DNA microarray-based method to semi-quantify signal strength potentially allows the determination of the most dominant haplotype.

By the successful application of our microarray system for molecular drug resistance monitoring in several sites over three years in Papua New Guinea, we could demonstrate that

standardised and comparable data could be produced at an affordable price. The flexibility of the system facilitates prompt inclusion of newly identified point mutations associated with parasite resistance in the future. Hence, the method offers unmatched capacity to provide evidence-based data on the dynamics of parasite resistance against antimalarial drugs in a cost-effective way.

This platform can also be widely applied and adapted with ease to other genotyping tasks requiring parallel SNP analyses. These include for instance molecular markers for other important host-determinants for antimalarial treatment response, such as pharmaco- or immunogenetic markers (Meyer, 2004; Plebanski *et al.*, 2002), as well as genetic determinants of susceptibility to disease (Williams, 2006).

### **3.2 The role of molecular markers in predicting treatment response**

Though the relevance of genetic determinants of drug resistance to several drugs has been confirmed *in vitro*, a straightforward association of molecular markers with *in vivo* treatment response has rarely been found (Bloland, 2001). Epidemiological studies dealing with that subject were predominantly clinical drug efficacy trials and frequently applied the two following designs:

- i) Determination of drug resistance-associated SNPs in parasites circulating in pre-treatment samples and the analysis of their relationship with treatment response, and/or
- ii) Comparison of parasite genotypes in samples before and after treatment in order to trace the genetic changes in the parasite gene(s) upon selective pressure exerted by the respective drug under investigation.

These studies almost exclusively focused on the investigation of single drug classes as well as the analysis of single genes or markers. Hence, data on the association of multiple markers for different drug classes and studies evaluating their association with *in vivo* response to combination therapy are still scarce. Nevertheless, more recent molecular methods, such as our novel DNA microarray-based technique, greatly facilitate the parallel assessment of parasite resistance markers of several drug classes at once. The application of this method allows a more comprehensive assessment of the genetic resistance background of the parasite and hence, a more accurate elucidation of the specific role of different molecular markers

(i.e., direct mediation of resistance, modification of resistance, compensation for functional constraints or fitness costs) (Bray *et al.*, 2005; Duraisingh & Cowman, 2005; Hayward *et al.*, 2005; Walliker *et al.*, 2005).

In our studies, we analysed the genetic profile of parasites collected from pre-treatment samples of 206 malaria patients attending two health facilities in PNG with known clinical and parasitological outcomes after treatment with AQ or CQ plus SP (Chapter 2). The analysis of twenty-four key markers in *P. falciparum* *mdr1*, *pfcr1*, *pfdhfr* and *pfdhps*, revealed a genetic background that was consistent with high-level resistance to CQ. In addition, mutation rates of molecular markers for pyrimethamine-resistance in *pfdhfr* were already high and sulphadoxine resistance-related SNPs in *pfdhps* started to emerge (Chapter 4).

Our results showed that the strongest independent predictors for treatment failure with AQ or CQ plus SP were *pfmdr1* N86Y and *pfdhps* A437G. Mutations found in CQ related markers (i.e., *pfcr1* K76T, A220S, N326D, and I356L) did not help to increase the predictive value, the most likely reason being that these mutations are almost fixed in the parasite population in PNG. Though mutations in SP related markers *pfdhfr* S108N and C59R were not associated with treatment failure, they increased the predictive value of *pfdhps* A437G. Consistent with the hypothesis that the genetic parasite resistance background in part determines *in vivo* treatment response, the most significant association with treatment failure with AQ or CQ plus SP was seen in patients who harboured the most highly mutated combined *pfmdr1/pfcr1/pfdhfr/pfdhps* genotype (Chapter 4).

Our results are in agreement with previous data that have shown that the genetic drug resistance profile usually precedes the observation of *in vivo* resistance (i.e., mutation rates for resistance alleles are higher than corresponding treatment failure rates) (Djimde *et al.*, 2001b; Kublin *et al.*, 2002). Furthermore, mutations that have reached almost fixed levels in the parasite population have already been shown to be unsuitable as markers for resistance (Dorsey *et al.*, 2001; Mayor *et al.*, 2001; Rallon *et al.*, 1999).

A significant association between *pfdhps* A437G and treatment response with CQ plus SP was found in a recent study conducted in Laos (Berens *et al.*, 2003). In contrast, the K540E mutation in *pfdhps* was shown to be a better indicator of treatment failure with the same combination regimen in Uganda (Dorsey *et al.*, 2004). These contrasting reports clearly reflect that, apart from the genetic background of the parasite, there are several other factors which determine individual treatment outcome, the most important being the pharmacokinetic and pharmacodynamic properties of the drug(s) and the level of acquired immunity of the host

(White, 1998). Furthermore, former drug pressure as a major driving force for the development and spread of parasite resistance and hence, the determination of the genetic resistance profile of the parasite, is different between epidemiological settings. Therefore, the relevance of molecular markers in predicting treatment response varies between different regions accordingly.

To summarize, extrapolation of results obtained in a given area to different epidemiological settings is risky, since both, important determinants of parasite resistance (e.g. drug pressure, population structure of parasites, transmission intensity, etc.) and host factors influencing treatment response (e.g. compliance to treatment, drug metabolism, transmission intensity as a major determinant for acquisition of immunity, etc.) are variable in different regions (Alifrangis *et al.*, 2003; Djimde *et al.*, 2003; Francis *et al.*, 2006; Staedke *et al.*, 2004).

As in the case of *P. falciparum*, the molecular monitoring of drug resistant *P. vivax* malaria would be an equally valuable complementary tool to *in vivo* studies, the most important reason being that the assessment of drug efficacy against vivax malaria is complicated by difficulties in clearly differentiating between treatment failures (true recrudescences originating from asexual blood stage parasites), relapses (red blood cell infection originating from hypnozoites) and newly acquired infections.

Orthologous genes of *pf dhfr*, *pf dhps*, *pf crt*, and *pf mdr1* have been found in *P. vivax* (i.e., *pvdhfr*, *pvdhps*, *pvcg10* and *pvmdr1*, respectively). Though there is sound *in vitro* evidence for their role in conferring parasite resistance, their association with *in vivo* drug response has only been demonstrated for important SNPs in *pvdhfr* and *pvdhps* (Chapter 7).

In the current study, we investigated the relationship between drug resistance markers in *pvdhfr* and *pvmdr1* in pre-treatment samples from patients with a *P. vivax* mono-infection and therapeutic outcome with the newly introduced combination regimen in PNG. Thereby, we identified a novel molecular marker in *pvmdr1* to be associated with *in vivo* response to AQ or CQ plus SP. We also measured high prevalence rates of mutated key markers in *pvdhfr*. As with falciparum malaria, an infection with the quadruple mutant *pvdhfr* 57L+58R+61M+117T plus *pvmdr1* mutation 976F represented a significant risk of treatment failure with AQ or CQ plus SP. Our results demonstrating that the observed difference in failure rates between sites was reflected in the corresponding genetic drug resistance profile of the respective parasite populations, could further confirm the usefulness of our proposed marker set (i.e., *pvdhfr* F57L/I, T61M, and S117T/N plus *pvmdr1* Y976F) as a supplementary monitoring tool for vivax malaria (Chapter 7).

Taken together, our results emphasize again that a careful baseline assessment of resistance markers including the investigation of their relationship with treatment response is important for the identification of appropriate marker sets in a given area. Moreover, though the internal validity of results from a given study may be good for the area where the assessments have been made, external validity may be heavily compromised because epidemiological characteristics (e.g. malaria endemicity, transmission intensity, drug use patterns, etc.) which are known to affect the development and spread of parasite resistance, can vary considerably between different geographic regions. Moreover, the interaction of these factors as well as their impact on the development and spread of resistance are still controversial issues (Ariey & Robert, 2003; Hastings, 2003; Hastings & D'Alessandro, 2000; Mackinnon & Hastings, 1998; Talisuna *et al.*, 2002a; Talisuna *et al.*, 2003a). However, though once identified molecular markers are not absolutely reliable indicators for individual patient outcome, they may represent a useful public health tool for longitudinal monitoring of antimalarial resistance on population level (Sibley & Hunt, 2003).

### **3.3 The role of molecular markers in monitoring parasite resistance**

Though molecular markers have been advocated as a rapid means for the surveillance of resistance in order to provide timely and evidence-based information for policy formulation, they are still rarely used for this purpose (Plowe, 2003, Plowe, 2005). This is most likely due to the lack of simple models that produce appropriate indicators to be applied in different epidemiological settings for various drugs and combinations.

One possible model for CQ resistance, based on a genetic resistance index (GRI, ratio between prevalence of resistance genotype and prevalence of *in vivo* resistance) and a genetic failure index (GFI, ratio between prevalence of resistance genotype and incidence of *in vivo* treatment failure) has been proposed by Djimde *et al.* (2001a). Thereafter, similar models based on key molecular markers for SP resistance have been validated (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003). These models suggest that if GRIs and GFIs are stable over time (i.e., corresponding changes in parasite resistance and *in vivo* response), once established indices could be used for surveillance of resistance and molecular surveys could then provide a new epidemiological tool to extend the coverage of drug resistance monitoring beyond selected sentinel sites (i.e., include remote areas) (Plowe, 2003).

Though the analysis of point mutations on population level and the establishment of correlations between the molecular drug resistance profile in parasites and *in vivo* outcome

could give a more comprehensive appraisal of the status and longitudinal dynamics of resistance, only a few studies have investigated the correlation between population-based molecular data and the level of clinical failure in health facilities of the same region. (Djimdé *et al.*, 2001a; Djimdé *et al.*, 2001b; Talisuna *et al.*, 2002b; Talisuna *et al.*, 2003b) Furthermore, these studies have mainly focused on single loci conferring resistance to a single drug class (i.e., CQ or SP).

The current study in PNG aimed to bring together molecular data from community-based surveys and data derived from health centre-based studies collected over a time period of three years. After we had demonstrated in a first step, that there was no difference between the genetic drug resistance profile in clinical and community samples for two sites that are different with regard to epidemiological characteristics as well as drug use patterns (Chapter 5), we investigated the potential of molecular marker frequencies and genotype patterns assessed on community level to reflect the longitudinal trends of failure rates with AQ or CQ plus SP at the corresponding health facilities. We finally evaluated the usefulness of the GFI model for surveillance of resistance under the current first-line regimen in PNG (Chapter 6).

Treatment failure rates with the current combination regimen were reflected in the corresponding genetic resistance pattern of parasites from community samples. Frequencies of mutated alleles of AQ/CQ relevant markers in *pfprt* and *pfmdr1* were high and did not show significant changes over time. Also mutant allele frequencies in the pyrimethamine relevant gene *pfdhfr*, which were still moderate, were not significantly variable over time. In contrast, mutations in *pfdhps*, involved in resistance to sulphadoxine, were still low, but had started to emerge with variable degree at all three sites. The opposing longitudinal trends in clinical response observed at two sites (i.e., decreasing in Karimui and increasing in the Wosera) were best reflected by the frequencies and genotype patterns of mutations in SP relevant genes *pfdhfr* (S108N plus C59R) and *pfdhps* (A437G). Though the GFI based on the prevalence of the combined *pfdhfr* S108N+C59R plus *pfdhps* A437G genotype was most reliably predicting longitudinal *in vivo* trends at a given site, it was less useful for the comparison of different sites (i.e., different epidemiological settings) at a given time point.

On one hand, these data further corroborated our previous findings that demonstrated this genotype to be the best predictor for treatment failure on individual level (Chapter 4) and suggest this genotype to be a valuable marker for the level of clinical failure on population level over time at a given site. On the other hand, our results are not consistent with African studies that had shown the *pfdhps* genotype not to be indicative for treatment failure with SP



monotherapy in Ghana and Tanzania (Mockenhaupt *et al.*, 2005b; Mugittu *et al.*, 2004; Mutabingwa *et al.*, 2001). However, other authors had suggested the quintuple mutation (triple *pfdhfr* 108+59+51 plus double *pfdhps* 473+540) as important predictor for treatment failure with SP in Uganda and Malawi (Kyabayinze *et al.*, 2003; Kublin *et al.*, 2002). Other studies had reported *pfdhps* mutations to be good predictors for unsuccessful treatment response to combination therapy with CQ plus SP (Berens *et al.*, 2003; Dorsey *et al.*, 2004).

These conflicting reports underscore the need for a careful baseline assessment of the molecular marker profile in parasite populations in a given epidemiological setting, the investigation of its relationship with *in vivo* treatment response, and the monitoring of its dynamics over time. They clearly indicate that former drug history is an important determinant of the genetic resistance background in parasites and that SP resistance may emerge and spread very differently according to whether SP was used as monotherapy or introduced as partner component in a combination regimen. In addition, other important parameters, such as drug use patterns as well as immunity related to transmission intensity (Djimé *et al.*, 2003; Francis *et al.*, 2006; Talisuna *et al.*, 2006) play a significant role in determining the level and spread of parasite resistance in a given area. Hence, indicators based on molecular data have to be considered with caution with regard to prior drug use and key epidemiological characteristics (e.g. malaria endemicity, intensity and seasonality of transmission, etc.) and interpreted in the local context where they had been assessed and will be used in the future.

The importance, that also other factors have to be considered in the validation of molecular markers and hence, the establishment of putative models for surveillance, was also reflected in our findings with the GFI model in PNG. In contrast to previous reports, which showed this indicator to be constant over time and postulated a stable relationship between the prevalence of *pfprt* K76T and therapeutic failure with CQ (Djimé *et al.*, 2001a), we found that GFIs based on CQ relevant genotypes were highly variable and did not reflect the corresponding *in vivo* failure trends, the most likely explanation being that a highly CQ resistant genetic background is quasi fixed in the parasite population in PNG. Likewise, GFIs based on prevalence rates of *pfdhfr* genotypes were also not able to reflect the corresponding *in vivo* trends, with frequencies of the most predominant double *pfdhfr* mutant (S108N+C59R) being stable over time (i.e., GFIs reflected an inverse *in vivo* trend). In contrast, GFIs based on the combined *pfdhfr/pfdhps* genotype have proven to be the best indicators for the longitudinal trends of failure rates observed at all the three sites.

To conclude, the currently suggested GFI model remains to be validated in other areas representing settings with wide variations in epidemiological characteristics and drug use history, as well as for different drugs and drug combinations. Molecular data on antimalarial parasite resistance from different continents and/or countries are rich, but they show that the development and suggestion of a universal approach seem to be difficult. However, the appraisal we used in the current study (i.e., community-based surveys and the analysis of the whole array of drug resistance-associated molecular markers), which provides a tool for a more comprehensive assessment of the situation in a given area, may open the avenue to make better decisions on molecular monitoring approaches in a given area.

Fortunately, mutations in *pfATPase6*, the gene encoding a putative target for artemisinin derivatives (Eckstein-Ludwig *et al.*, 2003; Jambou *et al.*, 2005), were as yet not detected in PNG. This finding is important seeing that artesunate has already been officially introduced as partner drug with SP for second-line treatment against severe and treatment failure malaria, and a policy change to ACT as potential replacement option to AQ or CQ plus SP has to be taken into consideration in the near future.

Taken together, our *in vivo* and molecular data from PNG demonstrate 1) a highly mutated CQ resistance background, 2) a frequency of *pfdhfr* mutations consistent with a moderately pyrimethamine resistant phenotype, and 3) the emergence of key mutations in *pfdhps*. The latter was not surprising in view of former drug use history in PNG (Chapter 2). Furthermore, they suggest that SP, and more specifically sulphadoxine, is the effective component in the current first-line regimen and hence, molecular monitoring of resistance to this component is important under constant treatment policy in PNG. However, future marker sets will have to be adapted according to possible policy changes, such as the introduction of entirely new drug classes, as well as the cessation of drugs with unacceptably low levels of efficacy. Further monitoring of molecular markers of withdrawn drugs is an important issue, especially in the view of the limited drug armamentarium against malaria. Recent studies showed that the cessation of CQ was followed by re-emergence of CQ sensitive *P. falciparum* (Kublin *et al.*, 2003). Moreover, increasing clinical efficacy trends were reflected in the corresponding drug resistance profile of the parasites (i.e., disappearance of the CQ resistant *pfcr* K76T genotype) (Mita *et al.*, 2003). These observations are promising and the possibility of rotating the limited number of safe, effective and affordable antimalarials could be considered (Laufer & Plowe, 2004).

### 3.4 Implications for future research and recommendations

Though a vast amount of molecular data on antimalarial parasite resistance has been produced during the last two decades, there are many questions and issues which have to be addressed in the future. Not only for a better understanding of the molecular mechanisms conferring parasite resistance to several drug classes and combinations, but also to develop and validate simple but comprehensive models, so that molecular data can be used as a source for establishing indicators for resistance. The most important issue being that these indicators can be used by health authorities in the decision-making process on drug policy in malaria endemic countries.

The present study has highlighted again several critical and in part controversial issues of current methods of *in vivo*, as well as molecular assessment of drug resistant malaria, the most important being:

- The drawbacks of the current *in vivo* drug efficacy study protocol, especially the lack of consensus on how genotyping data for the differentiation between recrudescences and new infections are generated and analysed.
- The difficulties in comparing molecular resistance data in general, and more specifically in extrapolating the findings from one epidemiological setting to another. The main reasons being:
  - The absence of standardised protocols for a comprehensive baseline assessment of the genetic drug resistance profile in parasites that enables more accurate selection of relevant markers for subsequent monitoring, as well as better comparability of data from studies conducted in different areas.
  - Limited information about the progression of parasite resistance in areas which differ with regard to several characteristics, such as demographic and ecological parameters, malaria endemicity, level and pattern of disease transmission, and drug usage.
- The lack of simple models that may provide reliable indices based on molecular data in order to be helpful to several potential users, such as scientist, health care professionals and policy makers.

Some of the questions will have to be addressed by ongoing activities in basic research investigating:

- I) The molecular mechanisms underlying anti-plasmodial action of several drug classes. These include old and new generation aminoquinolines and antifolates, as well as artemisinin derivatives and entirely new compounds that will be discovered in the future.
- II) The molecular basis determining parasite resistance to different drug classes. Future *in silico* and *in vitro* research will lead to the identification and characterisation of other genes, as well as other genetic modification and/or epistatic interactions between genes to be involved in conferring parasite resistance.

Further insight into the role and relevance of molecular markers in predicting *in vivo* treatment response, as well as the validation of their usefulness as a public health tool for monitoring resistance will be obtained by the following studies:

- I) The investigation of genetic determinants of parasite resistance in the field, an important issue being the elucidation of the role of the molecular parasite background in determining *in vivo* response. This will also include the development of proxy indicators for other determinants of resistance, such as host drug metabolism, host immunity, transmission intensity and drug pressure, which have to be considered in the analysis.
- II) Validation of the applicability of presently proposed marker sets for different drug classes and combination regimens (e.g. *pfcr* K76T for CQ, triple *pfdhfr* (S108N+C59R+N51I) or quadruple *pfdhfr/pfdhps* (plus A437G+K540E) for SP as suggested in certain areas in Africa, or triple *pfdhfr/pfdhps* (S108N+C59R plus A437G) for AQ/CQ plus SP as suggested in the present study) in different epidemiological settings.
- III) The search for new approaches in dealing with the problem of multiclonal *P. falciparum* infections. These include the assessment of the relationship between mutations within and between genes (linkage disequilibrium), as well as the development of novel probabilistic models possibly providing estimates of genotypes and/or haplotype frequencies in a patient sample. Further refinement of

our novel DNA microarray-based technology, which already allows a semi-quantitative assessment of several SNPs, combined with a genotyping method capable of quantifying MOI, may potentially enable the determination of the most dominant resistance genotype/haplotype in a patient sample in the future.

- IV) The molecular analysis of archived blood samples which will provide valuable information about 1) genetic changes in the parasite even after a new drug has been introduced and/or a previously inefficacious drug has been withdrawn, and 2) the time lag between the occurrence of parasite resistance and *in vivo* treatment failure which will allow better predictions about the likely progression of resistance in a given area.
- V) The validation of currently suggested models and concepts for molecular monitoring of antimalarial resistance (e.g. GFIs) in various epidemiological settings. Previous studies evaluating the GFI model, including the investigations in the present study, indicate this model to be too simplistic. Hence, additional parameters for other determinants of parasite resistance (e.g. drug pressure) and the level of host immunity (e.g. transmission intensity) may have to be taken into account in future public health models.

More specific recommendations for future research and resistance monitoring activities in PNG include:

- 1) Selection and comparative evaluation of potential replacement options, preferably artemisinin-based combination regimens (with the current first- and second-line regimens being included as control arms in the studies). Apart from safety and efficacy, important aspects to consider include pre-existing and putative cross-resistance to potential partner drugs, acceptability in the communities, practicability within the health infrastructure in PNG, and costs.
- 2) Molecular monitoring of parasite resistance at representative sentinel sites. Under constant drug policy in PNG, we suggest to use the marker sets for *P. falciparum* and *P. vivax* proposed in the present study. In view of artesunate being used as partner drug with SP in the current second-line regimen, we strongly recommend to include the currently proposed *pfATPase6* SNPs as additional markers. However, marker sets will have to be adapted according to future drug policy change(s) (e.g. inclusion of 4-aminoquinoline markers after withdrawal of CQ and AQ).

- 3) After careful evaluation in PNG, our community-based molecular monitoring approach has been shown to be feasible under current treatment strategy in the areas where the assessment has been done. Therefore, molecular monitoring using this approach and applying the proposed marker set can be recommended for the short-term (i.e., under constant treatment policy) in these areas. However, the validity of this community-based approach has to be proven for other drug(s) and drug combinations in different epidemiological settings, especially with regard to differences in transmission intensity and drug use patterns, before a similar approach can be propagated for other areas within PNG, as well as in other malaria endemic countries.

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## **Appendix I**

### **Malaria control in Papua New Guinea results in complex epidemiological changes**

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**Abstract**

With a renewed interest in large-scale malaria interventions knowledge about the possible long-term effects of such interventions on the nature of malaria transmission is essential. We document complex changes in malaria epidemiology over the last 40 years associated with changing malaria control activities in Karimui, an isolated area in Papua New Guinea. An initially equal distribution of *P. falciparum*, *P. vivax* and *P. malariae* changed to currently 68% *P. falciparum*, after passing through a phase of transitory *P. vivax* dominance, when control started to fail. Initial drops in malaria prevalence proved difficult to sustain and present post-control levels are significantly higher than pre-control levels. The example of Karimui indicates that unsustained control can lead to changes in malaria patterns that may leave a population worse off.



## Introduction

Renewed awareness of the immense human and economic costs of malaria has brought malaria control once again to prominence on the international public health agenda. There has been extensive discussion of the possible effects of malaria interventions on protective immunity and patterns of morbidity<sup>1,2</sup>, but less attention has been given to the long-term effects on malaria transmission itself, especially in areas outside Africa that have complex malaria patterns.

In Africa, a massive resurgence of malaria was seen after cessation of control in many areas<sup>3</sup>, but the absence of significant levels of non-*falciparum* malaria preclude investigation of differential control effects on individual malarial species. Following the cessation of DDT spraying in Sri Lanka in 1964, *P. vivax* quickly re-established itself causing major epidemics<sup>4</sup>. Control was reintroduced and, following the switch from vector control to exclusive treatment of malaria cases and the first reports of chloroquine resistance, a steep rise in *P. falciparum* cases was observed<sup>4</sup>, although *P. vivax* remained the dominant parasite<sup>5</sup>. In areas of Asia and South America with ongoing malaria control programs, for instance Thailand and Brazil, a shift from *P. falciparum* to *P. vivax* preponderance occurred in the last 20 years<sup>6,7</sup>, despite rising levels of drug resistant *P. falciparum*. The contrary trend of *P. falciparum* replacing *P. vivax* was, however, observed in parts of India with high levels of drug and insecticide resistance<sup>8</sup>. These patterns indicate complex relationships between control activities and the transmission of different malaria species in different parts of the world.

In Papua New Guinea, where all 4 human *Plasmodium* species and a multitude of vectors occur, the malaria control program that lasted from 1960 to the early 1980s was associated with a notable shift from *P. vivax* to *P. falciparum* predominance<sup>9,10</sup>. In Karimui, an isolated area without road access, located approximately 1000 m above sea-level (settlement range 900-1200 m) to the south of the main highland cordillera, control started later and lasted longer than elsewhere in PNG. Good, detailed malariological data from pre-control (1965,<sup>11</sup>), early into control (1971,<sup>12</sup>), breakdown of control (1981,<sup>13</sup>) and current, post-control times (2001/02<sup>14</sup>) are available from a series of large malaria surveys in this area. Furthermore, the history of control is relatively well documented<sup>14</sup>. This offers an exceptional opportunity to investigate changes in malaria epidemiology in relation to malaria control activities.

## Materials & Methods

This paper used results from four major malaria surveys, conducted between 1965 and 2002, in the Karimui and Daribi area on the Karimui Plateau, South Simbu. These surveys published in reports or scientific paper contained detailed description of sample locations and population and presented data in ways that allowed collating samples that were comparable in areas and age groups covered. A number of other malaria surveys, in particular from the control period, could not be used for this comparison as they lacked sufficient detail and/or documentation. Most were only available from field research note from one author (R. Hide) and either only covered parts of the Karimui area or did not contain sufficient detail on populations covered. The comparison was thus restricted to the 4 published surveys.

Following an initial malaria mass blood survey of the Karimui area in 1962, a major pre-control survey was conducted in August 1965 by the Malaria Control Service and included 3937 people in both Karimui and Daribi census districts (CD)<sup>11</sup>. Control measures began in early 1968, and included indoor residual spraying with DDT (IRS, two spray rounds annually) and mass drug administration (CQ and pyrimethamine, administered during the spray rounds from 1968 to 1970 only, Figure 1). Regular mass blood surveys were carried out twice a year to assess control efficacy, and a scientific assessment of the malaria situation under control was conducted in October-November 1971<sup>12</sup>. The 1971 study assessed 978 people on the Karimui Plateau.

A decade later, following reports of high levels of child malnutrition, in-depth epidemiological surveys were carried out in Karimui during August-September 1981 by members of the Simbu Land Use Project, the Provincial Department of Health and the PNGIMR. The aim of these surveys was to determine the prevalence of malnutrition, malaria and intestinal parasites<sup>13</sup>. Malarial infections and spleen rates were assessed in a total of 1591 individuals from 7 villages in Karimui and Daribi CD. Twice-yearly spraying was continued until 1978, when control was scaled back. Spraying continued at irregular intervals and decreasing coverage until the early 1980s (<sup>15</sup>, Figure 1). The 1981 survey thus coincided with the period of failing control characterised by erratic interventions and decreasing coverage and effectiveness.

In 1984, vector control was officially abandoned and until 2002 treatment of all presumptive malaria cases with chloroquine and primaquine has been the mainstay of malaria control throughout PNG. Although insecticide treated bednets (ITN) have become increasingly available in PNG in recent years, they were virtually absent in Karimui villages. As part of a

larger study into the epidemiology of malaria throughout the PNG highlands, 765 individuals in 4 villages were examined for malarial infections and morbidity between July 2001 and May 2002<sup>14</sup>. In addition, a further 263 individuals were surveyed in December 2002 in 3 additional villages surrounding Karimui Station as part of an ongoing study in molecular markers of drug resistance. For the present analysis, results from the latter study were included with those from the wider 2001-2 survey.

Although the same villages were not included in each of the four main studies used here, all studies conducted cross-sectional community surveys across the same areas of the Karimui Plateau, and comprised all age groups in similar proportions. As original data was only available from the 2001/02 surveys, age categories were set to provide maximum correspondence between tabulations in the published studies. The data from the most recent surveys were then reanalysed to fit these categories. All comparisons between and within studies were done using Chi-square tests and logistical regression.

## Results

The first detailed survey in 1965, prior to control measures, found a malaria prevalence of 19.4% across Karimui, with similar amounts of *P. falciparum*, *P. vivax* and *P. malariae* present (Table 1 & Figure 1). After malaria control started in 1968, overall malaria levels were rapidly brought down to 7.1% by 1971, with *P. falciparum* dominating over *P. vivax* (PR: 4.5% vs. 2.8%,  $p = 0.05$ , Figure 1). *P. malariae* was permanently reduced. The age distribution of cases was little affected during the early phase of control, with parasite prevalence peaking at 1-4 yr both in 1965 and 1971 (Figure 2).

The faltering of the control program in the late 1970s and early 1980s resulted in a massive surge in malaria transmission. In 1981, overall prevalence had climbed over 30%, significantly exceeding pre-control levels (Table 1,  $p < 0.001$ ). The increase was strongest in *P. vivax* (PR: *Pf* 13.9%, *Pv* 15.8%, *Pm* 4.4%, Figure 1) and peak prevalence shifted to the 5-9yr (*Pv*) and 10-14 yr (*Pf*, *Pm*) age groups (Figure 2).

By 2001, some 20 years after the breakdown of control, the overall prevalence of malaria had not risen significantly ( $p > 0.5$ ). However, there has been a major shift in species composition (Table 1). While the overall prevalence of *P. vivax* decreased to pre-control levels (6.9%), the prevalence of *P. falciparum* increased to 22.1% and now accounts for 67.6% of infections. Peak prevalence of parasitemia has shifted back towards younger age groups (Figure 1), most notable in *P. vivax* infections.

In the 2001/02 surveys there is a highly significant difference both in overall prevalence and age distribution of cases in relation to the distance of the surveyed village to a health centre. In villages within 1 hour walk of Karimui and Negabo health centres, overall prevalence was 22.5% compared to 35.6% in those further away ( $p < 0.001$ , Table 2) and age of peak prevalence was significantly higher ( $X^2 = 12.6$ ,  $df = 3$ ,  $p = 0.006$ ). Such differences were not observed ( $p > 0.2$ ) in the 1981 surveys, with overall prevalence of 33.6% and 36.0% in the 1-9 year and 24.0% and 20.2% in the over 15 year age groups in villages within or beyond 1 hour walking distance from the nearest health centre, respectively.

## Discussion

The data from Karimui not only show that the impressive initial reductions in malaria transmission achieved by control measures were difficult to sustain, but that the epidemiology of malaria in the area has been significantly changed over the course of these interventions.

The slight shift to *P. falciparum* early during the control period was probably due to mass drug administration, as has been seen in other parts of the country<sup>16</sup>. Surprisingly, the roles were reversed when the control efforts were breaking down. Between 1971 and 1981, irregular spraying and cessation of mass drug administration favoured *P. vivax* transmission. Several factors may have contributed to this change: its long-lasting liver stages, short extrinsic cycle and faster production of gametocytes<sup>17</sup> make *P. vivax* easier to transmit in marginal or fluctuating circumstances. Additionally, prolonged DDT spraying elsewhere in PNG led to a shift in vectors towards early- and outdoor-biting mosquitoes<sup>18</sup>. Early-biting mosquitoes in PNG tend to be younger and more likely to carry *P. vivax* sporozoites<sup>19</sup>, thus favouring transmission of *P. vivax*.

The resurgence of malaria following collapse of regular control was very rapid. Mass blood surveys in the same areas conducted by the malaria control services (usually at time of spray activities) in the 6-18 months preceding the 1981 survey, found a significantly lower overall prevalence (14-15%) with a similar predominance of *P. vivax* as in the 1981 survey (Hide pers. comm.).

As elsewhere in PNG, *P. falciparum* became the dominant malaria species in Karimui after the complete cessation of vector control in the 1980s, while *P. vivax* fell back to pre-control levels (Figure 1) with a comparable age distribution (Figure 2). Indiscriminate use of 4-aminoquinolines in combination with poor compliance and the advent of resistant *P. falciparum*<sup>9</sup> are the likely reasons for this shift. Both result in poor clearance of infections and increased gametocyte production, thus fuelling *P. falciparum* transmission. Ongoing *in vivo* follow-up and molecular drug resistance markers studies show high levels of 4-aminoquinolines resistance in Karimui (Marfurt & Mueller, unpublished data). *P. malariae*, with the longest extrinsic cycle and still full susceptibility to 4-aminoquinolines, never fully recovered.

During the period that these changes in malaria epidemiology occurred, other significant changes in human-environment relations took place in the Karimui area that may have influenced malaria transmission. Most importantly, these included substantial population increase (a doubling between 1962 and 1990), and a shift in settlement pattern from dispersed

longhouses to centralised nucleated villages. These trends resulted in larger, more clustered areas of cultivation and human use. Such conditions favour vectors such as *An. punctulatus* that breed in open, sunlit pools<sup>18</sup> that result from agriculture and other human activity, thus increasing the chance of transmission..

It has been argued that a change to a sedentary, agricultural life style favoured the transmission of *P. falciparum* over *P. vivax* and contributed to the worldwide *P. falciparum* dominance<sup>20,21</sup>. It cannot be ruled out, therefore, that changes in population density, agriculture and settlement pattern at Karimui may have contributed to the dramatic shift towards *P. falciparum* following the collapse of control activities. It may also have helped to seal the fate of *P. malariae*, a parasite well adapted to endemicity in sparse and mobile human populations<sup>20</sup>.

Malaria control may also have affected immune status causing shifts in the age of peak prevalence. Early on, age distribution was little affected, but during the breakdown of control there was a clear shift towards older children, as shown by the 1981 survey. In the 20 years since cessation of vector control, peak prevalence of malarial infections has shifted back to younger age groups, except in areas with ready access to antimalarial drug treatment. This indicates, that 13 years of control, even if imperfectly done, reduced immunity in children. Similar shifts in distribution of malaria cases to older age groups with decreasing transmission were also seen in Africa where they were linked to increases in bednet coverage and changes in first line-treatment<sup>2</sup>.

This Karimui example demonstrates that, as elsewhere in areas with complex malaria patterns<sup>4, 5, 6, 7, 8, 9</sup>, major control interventions not only result in temporary reductions in levels of transmission but may be associated also with significant, unpredictable and possibly long-term shifts in malaria epidemiology. Such shifts might be especially marked if control is not properly maintained and, as in the case of Karimui and PNG in general, may leave an area worse off than before control. While the Karimui experience does not contraindicate renewed malaria control efforts, it highlights the importance of monitoring changes in malaria epidemiology, as well as the need to sustain successful interventions once started.

### **Acknowledgements**

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**Table 1:** Malaria prevalence rates (PR), species composition and spleen rates (SR) in Karimui from 1965-2002

Year	n	PR	Species composition			SR
			<i>P. f.</i>	<i>P. v.</i>	<i>P. m.</i>	
1965	3,937	764 (19.4%)	36.7%	34.6%	28.7%	-
1971	978 <sup>a</sup>	69 (7.1%)	56.2%	34.3%	9.6%	262 (26.8%)
1981	1,591	482 (30.3%)	40.7%	46.4%	12.9%	488 (30.7%)
2001/02	1,028	314 (31.2%)	68.4%	21.1%	10.5%	400 (38.9%)

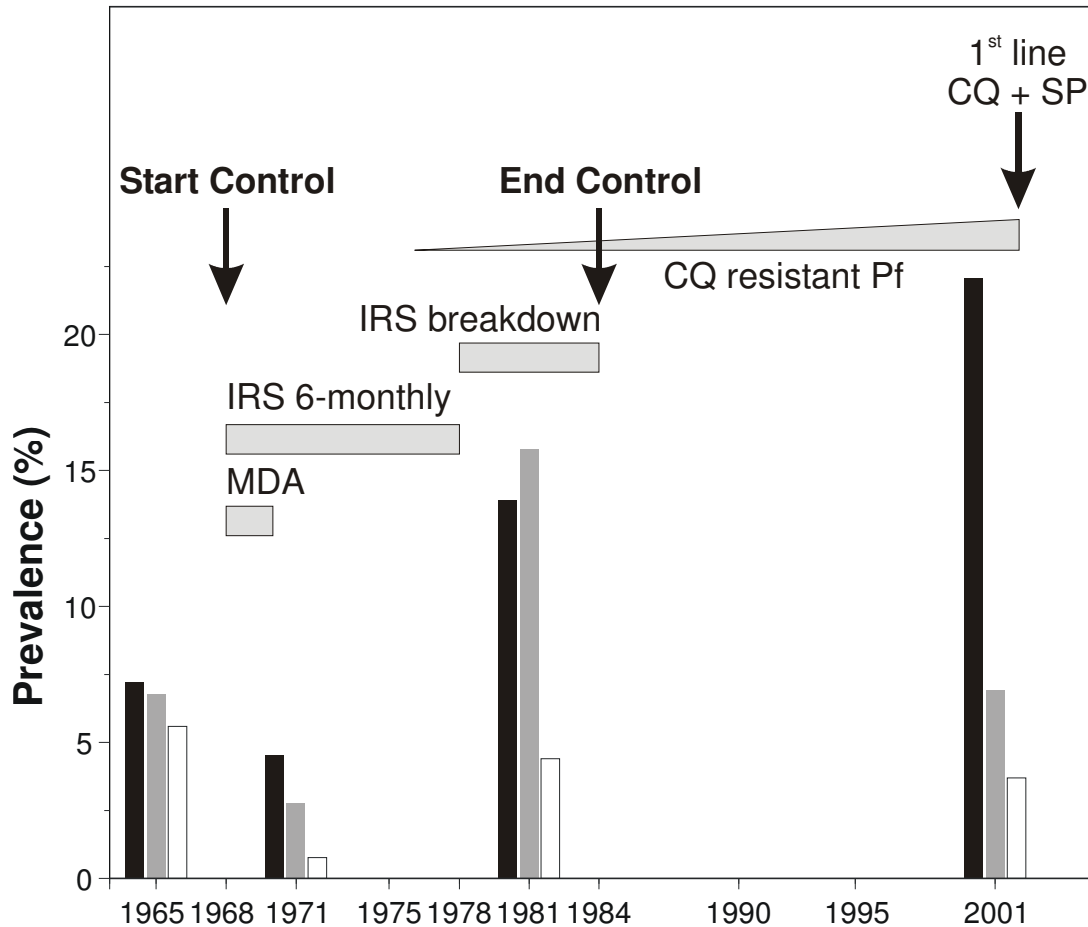
<sup>a</sup> Sample size for spleen rate 1084

**Table 2:** Age-specific prevalence of all malaria infection in 2001/02 surveys in relation to distance from nearest health centre

Age (years)	With 1 hour walk		> 1 hour walk	
	N	% pos	N	% pos
< 1	8	0.0	57	36.8
1 – 4	50	18.0	84	57.1
5 – 9	75	33.3	101	50.5
5 – 15	57	35.1	119	46.2
> 15	153	15.0	324	21.3
All	343	22.4	685	35.6

**Figure 1:** Changing prevalence of malarial infection in relation to control interventions in Karimui from 1965 – 2002.

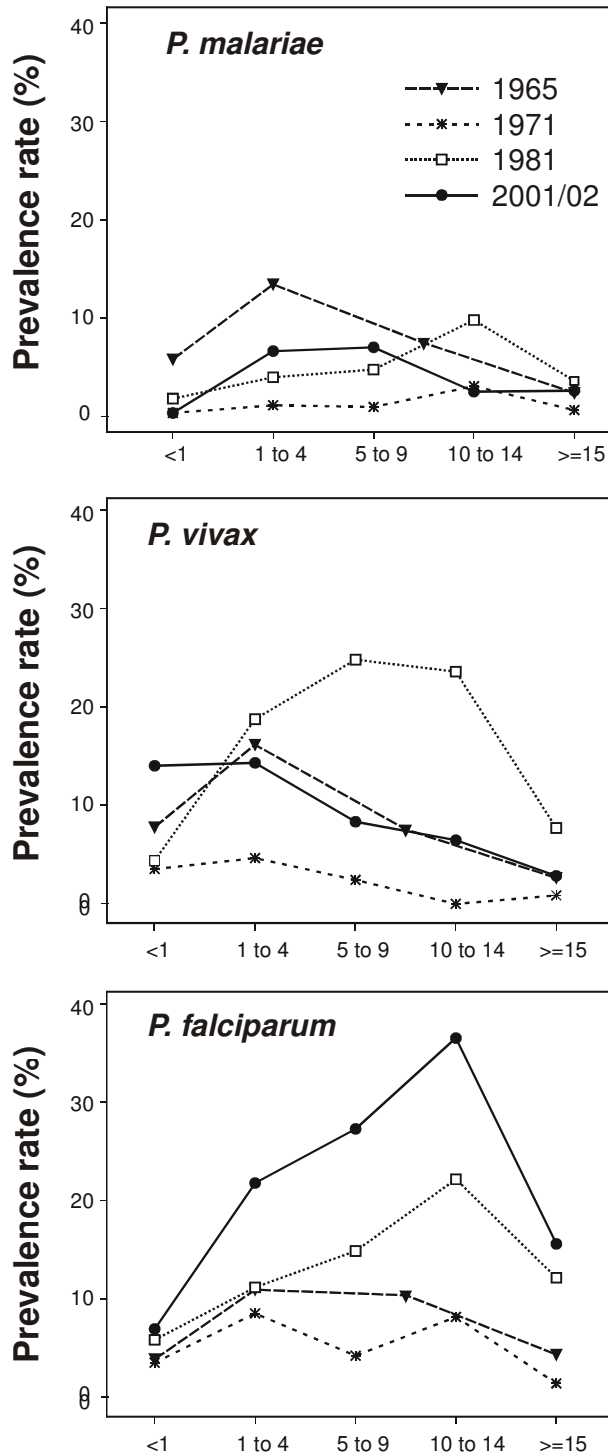
*P. falciparum* (Pf): black bars; *P. vivax*: grey bars, *P. malariae*: open bars. Abbreviations: IRS, indoor residual spraying with DDT; MDA, mass drug administration; CQ, chloroquine; SP sulphadoxine-pyremethamine.





**Figure 2:** Age-prevalence of different malarial infections in Karimui 1965 – 2002.

Data from 1965 survey did not allow differentiating into 5-9 and 10-14 age groups and date are thus given for age group 5-14 only.



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## **Appendix II**

### **Study forms and questionnaires**

**In vivo drug efficacy study:**

Date of health centre attendance:    (Day, Month, Year)

**Identification:**

ID number:

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex: F=female M=male	

*If a child is less than 6 months old, he/she must be excluded from the study!*

***Checklist for enrollement:***

<b>Exclusion criteria</b>	<b>Y/N</b>		
1. Age <6 months		4. Co-infection with any other disease	
2. Severe malnutrition (MUAC < 12 cm)		* 5. Current treatment with antibiotics	
3. Haemoglobin < 5g/dl		* 6. Antimalarial treatment within the last 28 days	
		7. Danger signs of complicated <i>Pf</i> malaria	

*\* Check the information with the health book of the patient.*

If there is 'YES' for any of these criteria, the patient **cannot be included** in the study!

**History of disease:**

Wanem dei sik i kamap ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tripela dei i go pinis; 5=Foapela dei i go pinis; 6=Fivepela dei i go pinis)

Wanem kain sik i kamap	Skin hat o skin kol o skin guria	Y/N
	Het pen	Y/N
	Kus	Y/N
	Pekpek wara	Y/N
	Arapela kain sik:	Y/N

*If a patient reports **YES** for cough and/or diarrhoea, he/she must be **excluded** from the study!*

**Check the health book:**

Date of last antimalarial treatment:  (Day, Month, Year)

*If a patient had an antimalarial treatment within the last 28 days, he/she must be **excluded** from the study!*

**Clinical assessment:**

Weight	kg
Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Respiratory rate	/ min
Chest indrawing	Y/N
Spleen grade (Hackett's grading system)	0-5; 8=not possible; 9=not done
Other signs:	

*If there are clinical signs of the following conditions:*

- Severe malnutrition (MUAC < 12 cm)
- Febrile conditions due to any other disease
- Severe *P. falciparum* malaria

*the patient must be **excluded** from the study!*

**Haematology:**

Bleedcode:

Microtainer™: \_\_\_\_\_ Y/N

IsoCode® Stix: \_\_\_\_\_ Y/N

Haemoglobin:  .  g/dl

*If the **haemoglobin** reading is below 5.0 g/dl, the patient must be **excluded** from the study!*

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

**Drugs (1<sup>st</sup> dose) administered on DAY 0:**

The patient received: *(tick two if received in combination)*

Chloroquine	Y/N
Camoquin	Y/N
Primaquine	Y/N
Amodiaquine	Y/N
Fansidar	Y/N
Other drug:	Y/N

---

Please check on DAY 1:

**Drugs (2<sup>nd</sup> dose) administered on DAY 1:** ☐ Y/N

Chloroquine	Y/N
Camoquin	Y/N
Primaquine	Y/N
Amodiaquine	Y/N
Fansidar	Y/N
Other drug:	Y/N

Date of health centre attendance:    (Day, Month, Year)

**Identification:**

**ID number:**

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Asde yu kisim marasin pinis	Y/N
Nau yu sik	Y/N
Nau yu gat skin hot o skin col o skin guria	Y/N
Nau yu gat het pen	Y/N
Nau yu gat traut	Y/N
Nau yu gat pekpek wara	Y/N
Arapela kain sik:	Y/N

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Other signs:	

**Haematology:**

Bleedcode:

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

**Drugs (3<sup>rd</sup> dose) administered on DAY 2:**

The patient received: *(tick two if received in combination)*

Chloroquine	Y/N
Camoquin	Y/N
Primaquine	Y/N
Amodiaquine	Y/N
Fansidar	Y/N
Other drug:	Y/N

Date of health centre attendance:    (Day, Month, Year)

**Identification:**

**ID number:**

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Nau yu sik	Y/N
Nau yu gat skin hot o skin col o skin guria	Y/N
Nau yu gat het pen	Y/N
Nau yu gat traute	Y/N
Nau yu gat pekpek wara	Y/N
Arapela kain sik:	Y/N

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Other signs:	

**Haematology:**

Bleedcode:

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				



Date of follow-up:    (Day, Month, Year)

**Identification:**

**ID number:**

Kristen nem		Village	
Nem bilong papa		Haumas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Taim mipela kisim blut yu bin sik gen: ☐ Y/N

Sapos 'YES': ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tripela dei i go pinis)

Taim yu kisim las tablet yu gat skin hot oskin kol o skin guria: ☐ Y/N

Sapos 'YES': ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tripela dei i go pinis)

Taim yu kisim las tablet yu gat:

Het pen	Y/N
Traut	Y/N
Pekpek wara	Y/N
Arapela kain sik:	Y/N

Taim mipela kisim blut yu yet bin go gen long:

Ait post	Y/N
Helt Senta	Y/N
Haus Sik	Y/N

Sapos 'YES': Date:    (D, M, Y)

Yu bin kisim marasin long hap: ☐ Y/N

Yu bin kisim marasin long arapela ples: ☐ Y/N

Sapos 'YES': Stori long marasin: Drugs: \_\_\_\_\_

Full treatment: ☐ Y/N

***Data should be checked with the health book!***

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Other signs:	

**Haematology:**

Bleedcode: 

--	--	--	--	--	--	--	--

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

Date of follow-up:  (Day, Month, Year)

**Identification:**

ID number:

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Taim mipela kisim blut yu bin sik gen: ☐ Y/N

Sapos 'YES': ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tripela dei i go pinis; 5=Foapela dei i go pinis; 6=Fivepela dei i go pinis; 7=Sixpela dei i go pinis)

Taim yu kisim las tablet yu gat skin hot oskin kol o skin guria: ☐ Y/N

Sapos 'YES': ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tripela dei i go pinis; 5=Foapela dei i go pinis; 6=Fivepela dei i go pinis; 7=Sixpela dei i go pinis)

Taim yu kisim las tablet yu gat:

Het pen	Y/N
Traut	Y/N
Pekpek wara	Y/N
Arapela kain sik:	Y/N

Taim mipela kisim blut yu yet bin go gen long:

Ait post	Y/N
Helt Senta	Y/N
Haus Sik	Y/N

Sapos 'YES': Date:  (D, M, Y)

Yu bin kisim marasin long hap: ☐ Y/N

Yu bin kisim marasin long arapela ples: ☐ Y/N

Sapos 'YES': Stori long marasin: Drugs: \_\_\_\_\_

Full treatment: ☐ Y/N

**Data should be checked with the health book!**

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Other signs:	

**Haematology:**

Bleedcode: 

--	--	--	--	--	--	--	--

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

Date of follow-up:    (Day, Month, Year)

**Identification:**

**ID number:**

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Taim mipela kisim blut yu bin sik gen: ☐ Y/N

Sapos 'YES': Insait tupela wik i go pinis \_\_\_\_\_ Y/N  
Insait onepela wik i go pinis \_\_\_\_\_ Y/N

Taim yu kisim las tablet yu gat skin hot oskin kol o skin guria: ☐ Y/N

Sapos 'YES': Insait tupela wik i go pinis \_\_\_\_\_ Y/N  
Insait onepela wik i go pinis \_\_\_\_\_ Y/N

Taim yu kisim las tablet yu gat:

Het pen	Y/N
Traut	Y/N
Pekpek wara	Y/N
Arapela kain sik:	Y/N

Taim mipela kisim blut yu yet bin go gen long:

Ait post	Y/N
Helt Senta	Y/N
Haus Sik	Y/N

Sapos 'YES': Date:    (D, M, Y)

Yu bin kisim marasin long hap: ☐ Y/N

Yu bin kisim marasin long arapela ples: ☐ Y/N

Sapos 'YES': Stori long marasin: Drugs: \_\_\_\_\_

Full treatment: ☐ Y/N

***Data should be checked with the health book!***

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Other signs:	

**Haematology:**

Bleedcode: 

--	--	--	--	--	--	--	--

**Malaria slide: compulsory! *Immediate reading in the afternoon!***

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

Date of health centre attendance:    (Day, Month, Year)

**Identification:**

**ID number:**

Kristen nem		Village	
Nem bilong papa		Hamas christmas bilong yu	
Nem bilong mama		Sex (F=female, M=male)	

**History of disease:**

Wanem dei sik i kamap ☐ (1=Nau; 2=Asde; 3=Asde bipo; 4=Tupela dei i go pinis; 5=Tripela dei i go pinis; 6=Foapela dei i go pinis; 7=Fivepela dei i go pinis)

Wanem kain sik i kamap

Skin hat o skin kol o skin guria	Y/N
Het pen	Y/N
Kus	Y/N
Pekpek wara	Y/N
Arapela kain sik:	Y/N

***Check with the health book:***

The patient received on DAY 0: (*tick two if received in combination*)

Chloroquine	Y/N
Camoquin	Y/N
Primaquine	Y/N
Amodiaquine	Y/N
Fansidar	Y/N
Artemether/Artesunate	Y/N
Other drug:	Y/N

**Clinical assessment:**

Axillary temperature	°C
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Spleen grade (Hackett's grading system)	0-5; 8=not possible; 9=not done
Other signs:	

**Haematology:**

Bleedcode:

--	--	--	--	--	--	--	--	--

**Malaria slide: compulsory! Immediate reading in the afternoon!**

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

Presumptive diagnosis: \_\_\_\_\_

**Drugs administered:**The patient received on DAY SICK: (*tick two if received in combination*)

Chloroquine	Y/N	Full treatment	Y/N
Camoquin	Y/N	Full treatment	Y/N
Primaquine	Y/N	Full treatment	Y/N
Amodiaquine	Y/N	Full treatment	Y/N
Fansidar	Y/N	Full treatment	Y/N
Artemether/Artesunate	Y/N	Full treatment	Y/N
Quinine	Y/N	Full treatment	Y/N
Other drug:	Y/N	Full treatment	Y/N

Sapos 'YES':

oral dose ☐ Y/N

Shot I.M. ☐ Y/N

Shot I.V. ☐ Y/N



**Community-based cross-sectional surveys: Household questionnaire**

Date:       (Day / Month / Year)

Village: \_\_\_\_\_

Province: \_\_\_\_\_

Village Number:

Province Number:

Household (HH) Number:

Name of the head of the HH: \_\_\_\_\_

Members of the HH:

Name	Date of birth (Day / Month / Year)	Reported age (Years or months)	Sex (F / M)	Present (Y / N)	If no (N), where?	ID number				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				
						XA				

Village number:   ID number: **XA**

Village: \_\_\_\_\_ Household number:

Kristen nem: \_\_\_\_\_ Sex: ☐ (F=female; M=male)

Pikinini: Nem bilong mama \_\_\_\_\_

Nem bilong papa: \_\_\_\_\_

Date of birth or approximate age:       (Day / Month / Year))

**Malaria questionnaire**

Insait long onepela wik I go pinis, yu bin sik? ☐ Y/N

- Sapos 'YES': Wanem kain sik i kamap?

Skin hat o skin kol o skin guria	Y/N
Het pain	Y/N
Kus	Y/N
Pekpek wara	Y/N
Arapela kain sik	Y/N
Wanem kain sik?	

- Wanem dei sik i kamap? ☐  
(0=Nau; 1=Asde; 2=Asde bipo; 3=Tripele dei i go pinis; 4=Foapela dei i go pinis; 5=Fivepela dei i go pinis; 6=Sixpela dei i go pinis; 7=Sevenpela dei i go pinis; 8=Etpela dei i go pinis; 9=Ninepela dei i go pinis; 10=Tenpela dei i go pinis; 13=not known)
- Wanem dei sik i go pinis? ☐  
(0=Nau; 1=Asde; 2=Asde bipo; 3=Tripele dei i go pinis; 4=Foapela dei i go pinis; 5=Fivepela dei i go pinis; 6=Sixpela dei i go pinis; 7=not finished; 9=not known)

Insait long onepela wik I go pinis, yu yet bin go gen long:

Ait post	Y/N
Helt Senta	Y/N
Haus Sik	Y/N

- Sapos YES': Yu bin kisim **malaria marasin** long hap? ☐ Y/N

Sapos YES': Wanem taim yu kisim las tablet \_\_\_\_\_ (Date)  
Wanem taim yu kisim las sut: \_\_\_\_\_ (Date)

If the **health book** of the patient is available, ☐ Y/N it should be checked for the following data:

Date of the last anti-malarial treatment:       (Day / Month / Year)

Treatment course: \_\_\_\_\_ Full treatment ☐ Y/N

Number of anti-malarial treatment courses during the preceding year:

No	Date (Day / Month / Year)	Course (Note two drugs if received in combination!)	Full course?
1			Y/N
2			Y/N
3			Y/N
4			Y/N
5			Y/N

Yu bin kisim **malaria marasin** long arapela ples?  Y/N

- Sapos YES': Wanem ples yu kisim malaria marasin:

Haus marasin	Y/N
Stoa	Y/N
Maket	Y/N
Ples dokta	Y/N
Wantoks	Y/N
Arapela ples	Y/N
Wanem ples?	

- Stori long marasin: Drugs: \_\_\_\_\_

### **Clinical assessment:**

Axillary temperature	°C
Respiratory rate	/ min
Current level of consciousness	(F = fully, D = drowsy, C = coma)
Chest indrawing	Y/N
Spleen grade (Hackett's grading system)	0-5; 8=not possible; 9=not done
Other signs Y/N	Specify:

### **Haematology:**

Bleedcode: XA  = ID number! Haemoglobin:  .  g/dl

Microtainer™:  Y/N Vacutainer™:  Y/N

### **Malaria slide: compulsory!**

Pf / 200 WBC				
Pv / 200 WBC				
Pm / 200 WBC				
Po / 200 WBC				
P. gam.				

*Whenever a subject is sick (clinical evidence and patient's report) or has a haemoglobin reading below 5.0 g/dl, she/he must be advised to go to the next health centre (assistance for transportation has to be provided!)*

## **INFORMED CONSENT**

(adapted from WHO/MAL/96.1077)

We are from the PNG Institute of Medical Research and we are interested in knowing how well the current treatment for malaria is working in this region of PNG. To do this, we are carrying out a study in which we are treating a group of children for malaria and then following them for 28 days to see if their infection is cured.

If you agree to participate the study, we would like to see your child 5 more times over the next 28 days (i.e. on day 2, 3, 7 14 and 28), so that we can monitor the progress of the treatment. At each visit your child will receive a full medical examination and we will take a small amount of blood by finger-prick to make blood smears to see if your child still has malaria parasites. It is very important that we see your child on these days, so if you feel that you cannot agree with this procedure, please let us know now.

Your participation is completely voluntary. If you do not want your child to participate in this study, he/she will receive treatment as usual at this health centre. Participation in this study will not cost you or your family anything. You may also withdraw your child from the study at any time and for any reason.

Your child will benefit from participating in this study because he/she will be closely followed over the next 28 days. If your child continues to suffer from malaria, he/she will receive an alternative treatment which will cure the illness. There will be someone here at the health centre every day so that, even on days between scheduled visits and on week-ends, you may bring your child for a check-up if you feel that he/she is ill.

Do you have any questions about the study?

## **INFORMED CONSENT**

(Tok Pisin)

Mipela wokman bilong PNG IMR i kam long wokim malaria research (risets). Mipela painim aut malaria marasin i wok long bodi long kilim i dai binatang bilong malaria or nogat.

Long stadi bai mipela i lukim ol pikinini antap long sixpela mun na moa, husait i save kisim bagarap long sik malaria.

Taim mipela enrolim yu long stadi na mipela i painim aut olsem yu gat sik malaria, em taim mipela i sekim blut bilong yu. Bai yu kisim tritmen long tripela dei. Bihain bai mipela lukim yu gen long las dei bilong marasin. Mipela bai mekim follow up insait onepela wik bihain, tupela wik bihain gen, na gen onepela mun bihain. Mipela bai kam bek gen long hauslain na kisim blut bilong yu long lukim sapos yu sik gen or olrait. Bihain long olgeta follow up, dispela wok bilong sekim yu i pinis.

As tingting bilong dispela wok em bilong helpim yu na olgeta kominiti insait long PNG na tu em bilong helpim gavman long kamapim gutpela marasin plen long daunim sik malaria.

Sapos yu wan bel orait, mipela bai enrolim yu long dispela stadi.



## **Appendix III**

### **Standard Operating Procedure:**

### **Analysis of malaria drug resistance markers using DNA microarray (chip) technology**

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## 1. DNA preparation

DNA is isolated from whole blood (anti-coagulated with EDTA) or red blood cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. [http://www1.qiagen.com/literature/protocols/QIAamp96DNABlood.aspx]

## 2. Amplification of target sequences

### 2.1 Primary PCR

#### 2.1.1 Primary PCR mix

Reagents	1 reaction	104 reactions (96 patients)	final conc.
H <sub>2</sub> O	30.0 µl	3120 µl	
10 x PCR buffer (without MgCl <sub>2</sub> )	5.0 µl	520 µl	1 x
dNTP mix (2mM)	5.0 µl	520 µl	200 µM
MgCl <sub>2</sub> (25mM)	6.0 µl	624 µl	3 mM
Primary PCR primer mix (10 µM each) #	1.0 µl	104 µl	200 nM each
Taq polymerase 5U/µl	0.50 µl	52 µl	0.05 U/µl
<b>Final volume</b>	<b>47.5 µl</b>	<b>4940 µl</b>	
DNA	2.5 µl		
<b>Final volume</b>	<b>50 µl</b>		

# Primary PCR primer mixes (sequence information: see Appendix):

1. P 1-1 (*pfmdr1* PCR I: P 1-1 for / P1-1 rev, 10 µM each in TE buffer)
2. P 3-1 (*pfmdr1* PCR II: P 3-1 for / P3-1 rev, 10 µM each in TE buffer)
3. P 5-1 (*pfdhfr* PCR: P 5-1 for / P5-1 rev, 10 µM each in TE buffer)
4. P 8-1 (*pf dhps* PCR: P 8-1 for / P8-1 rev, 10 µM each in TE buffer)
5. P 10-1 (*pf crt* PCR I: P 10-1 for / P10-1 rev, 10 µM each in TE buffer)
6. P 11-1 (*pf crt* PCR II: P 11-1 for / P11-1 rev, 10 µM each in TE buffer)
7. P 12-1 (*pf crt* PCR III: P 12-1 for / P12-1 rev, 10 µM each in TE buffer)
8. P 16-1 (*pf crt* PCR IV: P 16-1 for / P16-1 rev, 10 µM each in TE buffer)
9. P 17-1 (*pf ATPase6* PCR: P 17-1 for / P17-1 rev, 10 µM each in TE buffer)
10. P 18-1 (*pf crt* PCR V: P 18-1 for / P18-1 rev, 10 µM each in TE buffer)

#### 2.1.2 Primary PCR program

96 °C 180 sec

96 °C 30 sec

52 °C 90 sec

72 °C 90 sec

**20 cycles for clinical (symptomatic) samples**

**25 cycles for community (asymptomatic) samples**

Hold at 4 °C

## 2.2 Nested PCR

### 2.2.1 Nested PCR mix

Reagents	1 reaction	104 reactions (96 patients)	final conc.
H <sub>2</sub> O	60.0 µl	6000 µl	
10 x PCR buffer (without MgCl <sub>2</sub> )	10.0 µl	1000 µl	1 x
dNTP mix (2mM)	10.0 µl	1000 µl	200 µM
MgCl <sub>2</sub> (25mM)	12.0 µl	1200 µl	3 mM
Nested primer mix (10µM each)§	2.0 µl	200 µl	200 nM each
Taq polymerase 5U/µl	1.0 µl	100 µl	0.05 U/µl
<b>Final volume</b>	<b>95 µl</b>	<b>9500 µl</b>	
Primary PCR product	5.0 µl		
<b>Final volume</b>	<b>100 µl</b>		

§ Nested PCR primer mixes (sequence information: see Appendix):

11. P 1 (*pfmdr1* PCR I: P 1 for / P1 rev, 10 µM each in TE buffer)
12. P 3 (*pfmdr1* PCR II: P 3 for / P3 rev, 10 µM each in TE buffer)
13. P 5 (*pfdhfr* PCR: P 5 for / P5 rev, 10 µM each in TE buffer)
14. P 8 (*pfdhps* PCR: P 8 for / P8 rev, 10 µM each in TE buffer)
15. P 10 (*pfcr1* PCR I: P 10 for / P10 rev, 10 µM each in TE buffer)
16. P 11 (*pfcr1* PCR II: P 11 for / P11 rev, 10 µM each in TE buffer)
17. P 12 (*pfcr1* PCR III: P 12 for / P12 rev, 10 µM each in TE buffer)
18. P 16 (*pfcr1* PCR IV: P 16 for / P16 rev, 10 µM each in TE buffer)
19. P 17 (*pfATPase6* PCR: P 17 for / P17 rev, 10 µM each in TE buffer)
20. P 18 (*pfcr1* PCR V: P 18 for / P18 rev, 10 µM each in TE buffer)

### 2.2.2 Nested PCR program

96 °C 180 sec

96 °C 30 sec

52 °C 90 sec

72 °C 90 sec

**20 cycles for clinical (symptomatic) samples**

**25 cycles for community (asymptomatic) samples**

Hold at 4 °C

## 3. SAP (Shrimp Alkaline Phosphatase) digest of PCR products

### 3.1 Preparation of PCR products

By using a multichannel pipette, pool 10 µl of all the 10 nested PCR reactions from each patient into a new 96 well plate. Mix and centrifuge briefly. = **PCR pool plate**

Transfer 10 µl of each well into a new 96 well plate and add 90 µl of H<sub>2</sub>O to each well. Mix and centrifuge briefly. = **PCR pool plate 1:10**

## 3.2 SAP digest

**NOTE** Each PCR pool from **PCR pool plate 1:10** has to be SAP digested **in duplicate** because we have to perform **2 extension reactions** per patient!

### 3.2.1 SAP master mix

Reagents	1 reaction	104 reactions (48 patients)
H <sub>2</sub> O	4.0 µl	416 µl
10 x SAP buffer	1.0 µl	104 µl
Shrimp Alkaline Phosphatase (SAP) 1U/µl	2.0 µl	208 µl
<b>Final volume</b>	<b>7.0 µl</b>	<b>728 µl</b>
PCR pool 1:10	5.0 µl	
<b>Final volume</b>	<b>12.0 µl</b>	

- Using a multichannel pipette, transfer 5 µl of the PCR pool 1:10 to the **SAP plate**. Remember: you need **two wells per patient** (i.e., 1 SAP plate contains 48 patients).
- Add 7 µl of the SAP master mix to each well.
- Mix and centrifuge briefly, start SAP program.

### SAP plate

SAP plate	1	2	3	4	5	6	7	8	9	10	11	12
<b>A) SAP 1</b>	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
<b>B) SAP 2</b>	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
<b>C) SAP 1</b>	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
<b>D) SAP 2</b>	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
<b>E) SAP 1</b>												
<b>F) SAP 2</b>												
<b>G) SAP 1</b>											Patient47	Patient48
<b>H) SAP 2</b>											Patient47	Patient48

### 3.2.2 SAP program

SAP digest: 1 hour at 37 °C  
Inactivation of SAP digest: 15 min at 90 °C

**NOTE** This reaction is performed in a PCR machine.

## 4. Primer extension

### 4.1 Preparation of ddNTP mixes

Combination 1	Combination 2
ddATP <b>Cy3</b>	ddUTP <b>Cy3</b>
ddCTP <b>Cy3</b>	ddCTP <b>Cy3</b>
ddGTP <b>Cy5</b>	ddATP <b>Cy5</b>
ddUTP <b>Cy5</b>	ddGTP <b>Cy5</b>

- To get a **2.5  $\mu$ M** final concentration of ddNTP mixes (**Combination 1**, **Combination 2**), dilute Cy3- and Cy5-labelled ddNTP's stock solutions (100  $\mu$ M at -80°C) 1:40 in TE buffer:
  - 4 x 25  $\mu$ l 100  $\mu$ M ddNTP stock = 100  $\mu$ l. Add 900  $\mu$ l TE buffer = 1 ml 2.5  $\mu$ M ddNTP mix.
  - Make aliquots and store 2.5  $\mu$ M ddNTP mix at **-20°C**!

### 4.2 Preparation of extension primer mixes

Gene	Combination 1 (25 oligos)	Combination 2 (15 oligos)
<i>Pfdhps</i>	437, 540, 581, 613, 640	436, 613B, 645
<i>Pfdhfr</i>	16, 51, 59, 108, 164	108B, 164B
<i>Pfmd1r</i>	86, 184, 1034, 1042	1246
<i>Pfcrt</i>	72, 75B1, 152, 271, 326, 326B, 356, 356B	74, 76, 97, 163, 220, 371
<i>PfATPase6</i>	538, 769, 769B	574, 623, 683

- To get a **62.5 nM** final concentration of extension primer mixes (**Combination 1**, **Combination 2**), dilute extension primer stock solutions (10  $\mu$ M in TE buffer) 1:160 in TE buffer:
  - Combination 1**: 25 x 2  $\mu$ l = 50  $\mu$ l plus 270  $\mu$ l TE buffer
  - Combination 2**: 15 x 2  $\mu$ l = 30  $\mu$ l plus 290  $\mu$ l TE buffer
 Store extension primer mixes at **+4°C**!

### 4.3 Reaction mix **combination 1**

Combination 1	1 reaction	52 reactions (48 patients)	final conc.
H <sub>2</sub> O	1.6 $\mu$ l	83.2 $\mu$ l	
10 x Sequenase buffer	2 $\mu$ l	104 $\mu$ l	1 x
Extension primer mix <b>Combination 1</b> (62.5 nM)	2 $\mu$ l	104 $\mu$ l	6.25 nM
ddNTP mix <b>Combination 1</b> (2.5 $\mu$ M)	2 $\mu$ l	104 $\mu$ l	0.25 $\mu$ M
Thermo Sequenase (5U/ $\mu$ l)	0.4 $\mu$ l	20.8 $\mu$ l	0.1 U/ $\mu$ l
Final volume	8 $\mu$ l	416 $\mu$ l	

#### 4.4 Reaction mix **combination 2**

<b>Combination 2</b>	<b>1 reaction</b>	<b>52 reactions (48 patients)</b>	<b>final conc.</b>
H <sub>2</sub> O	1.6 µl	83.2 µl	
10 x Sequenase buffer	2 µl	104 µl	1 x
Extension primer mix <b>Combination 2</b> (62.5 nM)	2 µl	104 µl	6.25 nM
ddNTP mix <b>Combination 2</b> (2.5 µM)	2 µl	104 µl	0.25 µM
Thermo Sequenase (5U/µl)	0.4 µl	20.8 µl	0.1 U/µl
<b>Final volume</b>	<b>8 µl</b>	<b>416 µl</b>	

- Add 8 µl of the extension reaction mixes **Combination 1** and **Combination 2** to the SAP digested PCR products in the SAP plate = **EXTENSION plate** (Final volume = **20 µl**)
- Start extension program

#### EXTENSION plate

SAP→EXTENSION	1	2	3	4	5	6	7	8	9	10	11	12
<b>A) COMB 1</b>	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
<b>B) COMB 2</b>	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
<b>C) COMB 1</b>	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
<b>D) COMB 2</b>	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
<b>E) COMB 1</b>												
<b>F) COMB 2</b>												
<b>G) COMB 1</b>											Patient47	Patient48
<b>H) COMB 2</b>											Patient47	Patient48

#### 4.5 Primer extension program

94 °C    60 sec

94 °C    10 sec                    **35 cycles**  
 50 °C    40 sec

Hold at 4 °C

## 4.6 Denaturation

- Pool extension reaction mixes **Combination 1** and **Combination 2** with a multichannel pipette (Final volume = **40 µl**).
- Add 6 µl of denaturing solution:

0.5 µl 0.5M EDTA pH 8.0	
2.0 µl 10% SDS	
3.5 µl H <sub>2</sub> O	(Final volume: <b>46 µl</b> )
- Incubate at 94 °C for 60 sec
- Chill on ice for 2 min

## 5. Hybridisation of extended primers

### 5.1 Preparation of the spotted microarray

- Add 23 µl of the extension reaction mix on the chip
- Add 6 µl 20 x SSC to each well of the slide

### 5.2 Hybridisation

- Incubate the chip in a opaque humid chamber at 50 °C for 60-90 min

### 5.3 Washing procedure after hybridisation

1. 2x SSC + 0.2% SDS: 20 min at room temperature (RT)
  2. 2x SSC: 20 min at RT
  3. 2x SSC + 2% EtOH: 2 min at RT
- Dry the chip with compressed air and store at RT in the dark

## 6. Data acquisition and analysis

### 6.1 Base calling

Slides can be scanned by the use of any laser scanner.

#### IMPORTANT:

1. **Cy3 (wavelength: 532 nm) and Cy5 (wavelength: 635 nm) signals have to be acquired!**
2. **Single signal or combined signal images have to be stored as tif-files for further analysis!**
3. **File names have to include a unique study, slide, experiment and operator identification code**

### 6.2 Short guide for data analysis

- a)** Prior to analysis, patient/study identification numbers and their respective position on the slide(s) have to be entered using the galDesigner software:

1. Open **galDesigner** software
2. Load template = **malaria.sti**
3. Enter all 12 patient/study identification numbers in the respective fields on each slide
4. Save each slide (containing 12 patients) as separate **gal-file**

**Important:** file names have to include a unique **study, slide, experiment and operator** identification code

- b)** Slide images are analysed using the Axon GenePix® Pro (version 6.0) software ([www.axon.com](http://www.axon.com)):

5. Open **GenePix® Pro** software

"Image" menu:

6. Open image = **tif-file** (Ctrl+O))
7. Open array list = **gal-file** (Alt+Y)
8. Align array list (by using *Block Mode* and/or *Feature Mode*)
9. Analyse slide (creates image) (Alt+A)

"Results" menu:

10. Save results as GenePix Results Files = **gpr-file** (Alt+U)

"Report" menu:

11. Run report: by using the script: **Triplicates, ratios, with 6 parameters**
12. Start (creates the STI chip report)
13. Export the STI chip report as tab-delimited **txt-file**

**Important:** file names have to include a unique **study, slide, experiment and operator** identification code

- c)** Data are converted into a format compatible with any statistical package using fileConverter software:

14. Open **fileConverter** software
15. Transfer results as tab-delimited txt-files into the data folder
16. Run fileConverter
17. Rename and save the outTable.txt file in your personal data folder

## 7. Appendix

### 7.1 Buffers

- 500 mM EDTA pH 8.0
  - 180 mM phosphate buffer pH 8.0
  - 20 x SSC pH 7.0
  - 2 x SSC
  - 2 x SSC + 0.2% SDS
  - 2 x SSC + 2% EtOH
  - 10% SDS
  - 1 x TE buffer (= 10 mM Tris/HCl pH 8.0)
  - 10 mM Tris/HCl pH 7.4
1. Prepare all buffers/solutions according to the protocols in:  
*Molecular Cloning* (a laboratory manual; Sambrook J., Fritsch E.F. and Maniatis T. 2<sup>nd</sup> edition; Cold Spring Harbor Laboratory Press, 1989)
  2. Store all buffers at **RT**!

### 7.2 Reagents

- |  |                               |
|--|-------------------------------|
| • 10 x PCR buffer (=buffer B)              | Solis BioDyne, Tartu, Estonia |
| • 25 mM MgCl <sub>2</sub>                  | Solis BioDyne, Tartu, Estonia |
| • <i>Taq</i> polymerase (Firepol®; 5 U/μl) | Solis BioDyne, Tartu, Estonia |
| • dNTP mix (2mM each):                     |                               |

Dilute 100 mM stock solutions 1:50 in 10 mM Tris/HCl pH 7.4

dATP 100 mM	Amersham Biosciences: 272050
dTTP 100 mM	Amersham Biosciences: 272080
dCTP 100 mM	Amersham Biosciences: 272060
dGTP 100 mM	Amersham Biosciences: 272070

- |  |                                |
|--|--------------------------------|
| • 10 x SAP buffer                          | Amersham Biosciences: 70103    |
| • Shrimp Alkaline Phosphatase (SAP; 1U/μl) | Amersham Biosciences: 70092Z   |
| • 10 x Sequenase buffer                    | Amersham Biosciences: 93-79222 |
| • Thermo Sequenase (Termipol®; 5 U/μl)     | Solis BioDyne, Tartu, Estonia  |

**NOTE** Store all reagents at **-20 °C**!

- |                           |                      |
|---------------------------|----------------------|
| • Cy3 /Cy5 labelled ddNTP | Perkin Elmer: NEL999 |
|---------------------------|----------------------|

**NOTE** Store 100 μM ddNTP stock solutions at **-80 °C**!

### 7.3 Oligonucleotides

#### A. C-7 oligos (Spotting)

**500 μM** stock solutions in 180 mM phosphate buffer pH 8.0 (**aliquots at -20 °C**)

Operon (Amino **C-7 linker** at the 3' end!!!)

#### B. Extension oligos

**100 μM** stock solutions in TE buffer pH 8.0 (**aliquots at -20 °C**)

Operon (**HPLC**-purified!!!)



## 7.4 Sequence information

### NOTE:

- All primers and sequences are listed from the 5' to the 3' end of the sequence
- Sequences in *italic/underlined* denote flexible primer sequences (=flexi tag)
- Neg1-Neg4: negative control oligonucleotides (=conserved sequence stretches from the respective genes)

### 7.4.1 *Pfdhfr*

#### *Pfdhfr* primary PCR primer (Size PCR product: 677 bp)

P5-1 for: TTTATGATGGAACAAGTCTGC  
P5-1 rev: TAAATGATAAAATCCAATGTTGTAT

#### *Pfdhfr* nested PCR primer (Size PCR product: 637 bp)

P5 for: ACAAGTCTGCGACGTTTTTCGATATTTATG  
P5 rev: AGTATATACATCGCTAACAGA

#### *Pfdhfr* C-7 primer

DHFR Neg1 C-7: AAATATAAGAGATGTAAATATTTAAACAA  
  
16 C-7: CACATATGGCATAAATATCGAAAACGTC  
51 C-7: TACATTCCATGGTAATACTCCTTTATTTTC  
59 C-7: GTGCAGTTACAACATATGTGAA  
10/108 C-7: GCAGGGAAGCGGGAGCGAAACAGC  
11/108B C-7: AAAACGGGGCACAGCGGGCGGAA  
06/164 C-7: GGGAGAGCGCAGCAGGCAACAGAG  
07/164B C-7: GACCGCCACCAAGAACAGCACCGG

#### *Pfdhfr* extension primer

16: GACGTTTTTCGATATTTATGCCATATGTG  
51: GAAATAAAGGAGTATTACCATGGAAATGTA  
59: TTCACATATGTTGTAACGTCAC  
10/108: GCTGTTTCGCTCCCGCTTCCCTGCCAAAATGTTGTAGTTATGGGAAGAACAA  
11/108B: TTCCGCCGCGCTGTGCCCCGTTTTAAAGGTTTAAATTTTTTTGGAATGCTTTCCAG  
06/164: CTCTGTTGCCTGCTGCGCTCTCCCGGAAATTAAATTACTATAAATGTTTTATT  
07/164B: CCGGTGCTGTTCTTGGTGGCGGTCTTCTTGATAAACACGAACCTCCTA

***Pfdhfr* sequence (Accession number: J04643)**

P5-1 for

P5 for

P5 rev

P5-1 rev

C-7 and extension primer for *dhfr* 16, *dhfr* 51, *dhfr* 59, *dhfr* 108, *dhfr* 164

SNP

TTTATGATGGAACAAGTCTGC  
 ACAAGTCTGCGACGTTTTTCGATATTTAT  
 01 ATGATGGAACAAGTCTGC GACGTTTTTCGATATTTATGCCATATGTG C ATGTTGTAAGGTT  
 61 GAAAGCAAAAATGAGGGGAAAAAATGAGGTTTTTAATAACTACACATTTAGAGGTCTA  
 121 GGAAATAAAGGAGTATTACCATGGAAATGTA A TTCCTAGATATGAAATATTTT GTGCA  
 181 GTTACAACATATGTGAATGAA TCAAAATATGAAAAATTGAAATATAAGAGATGTAAATAT  
 241 TTAAACAAAGAACTGTGGATAATGTAAATGATATGCCTAATTCTAAAAAATTA CAAAAAT  
 301 GTTGTAGTTATGGGAAGAACA A CTGGGAAAGCATTCCAAAAAATTTAAACCTTT AAGC  
 361 AATAGGATAAATGTTATATTGTCTAGAACCCTAAAAAAGAAGATTTTGATGAAGATGTT  
 421 TATATCATTAAACAAAGTTGAAGATCTAATAGTTTTACTT GGGAAATTAAATTACTATAAA  
 481 TGTTTTATT A TAGGAGGTTCCGTTGTTTATCAAGAA TTTTGTAGAAAAGAAATTAATAAAA  
 541 AAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTTTTTCCAGAAATA  
 601 AATGAAAATGAGTATCAAATTATT CTGTAGCGATGTATATACT AGTAACAATACAACA  
 661 TTGGATTTTATCATTTA TAAGAAAACGAATAATAAAATGTTAAATGAACAAAATTTGTATA  
 721 AAAGGAGAAGAAAAAATAATGATATGCCTTTAAAGAATGATGACAAAGATACATGTCTAT  
 781 ATGAAAAAATTAACAGAATTTTACAAAAATGTAGACAAATATAAAATTAATTATGAAAAT  
 841 GATGATGATGATGAAGAAGAAGATGATTTTGTATTATTTAATTTAATAAAGAAAAAGAA  
 901 GAGAAAAATAAAAAATCTATACATCCAAATGATTTTCAAATATATAATAGCTTGAAATAT  
 961 AAATATCATCCTGAATACCAATATTTAAATATATTTATGATATTATGATGAATGGAAAT  
 1021 AAACAAAGTGATCGAACGGGAGTAGGTGTTTTAAGTAAATTCGGATATATTATGAAATTT  
 1081 GATTTAAGTCAATATTTCCATTATTAACACGAAGAAATTTTAAAGAGGAATTATT  
 1141 GAAGAATTGCTTTGTTTATTAGAGGAGAAACAAATGGTAATACGTTGTTAAATAAGAAT  
 1201 GTAAGGATATGGGAAGCTAATGGTACTAGGGAATTTTATAGATAATAGAAAATTTTCAT  
 1261 AGAGAAGTTAACGATTTAGGACCTATTTATGGTTTTCAATGGAGACATTTCCGGTGCTGAA  
 1321 TATACAAATATGTATGATAATTATGAAAATAAAGGAGTGGATCAATTAATAAATAATA  
 1381 AATTTAATTAATAATGATCCTACAAGTAGAAGAATTCTTTTGTGTGCATGGAATGTAAAA  
 1441 GATCTTGACCAATGGCATTACCTCCTTGTCATATTTTATGTCAGTTTTATGTTTTTCGAT  
 1501 GGGAAATTATCATGTATTATGTATCAAAGATCATGTGATTTAGGGCTAGGAGTACCTTTT  
 1561 AATATTGCTTCTTATTCTATTTTACTCATATGATTGCACAAGTCTGTAATTTGCAACCT  
 1621 GCGCAGTTTCATACACGTTTTAGGAAATGCACATGTTTATAATAATCACATTGATAGTTTA  
 1681 AAAATTCAACTTAACAGAATACCCTATCCATTCCCAACACTTAAATTAATCCAGATATT  
 1741 AAAAATATTGAAGATTTTACAATTCGGATTTTACAATACAAAATTATGTTTCATCATGAA  
 1801 AAAATTTCAATGGATATGGCTGCTTAA

## 7.4.2 *Pfdhps*

### *Pfdhps* primary PCR primer (Size PCR product: 756 bp)

P8-1 for: ATTTTGTGTTGAACCTAAACGTGCTGTTCA  
 P8-1 rev: CTTGTCTTTCCTCATGTAATTCATCT

### *Pfdhps* nested PCR primer (Size PCR product: 686 bp)

P8 for: TTGAAATGATAAATGAAGGTGCTAGT  
 P8 rev: CCAATTGTGTGATTGTCCA

### *Pfdhps* C-7 primer

DHPS Neg4 C-7: AACAAAAATTACATGATGAACAACAAAAAT  
 436 C-7: GGATTCTCCACCTATATCTATAA  
 437 C-7: TCCTTTTGTTATACCTAATCCAA  
 540 C-7: ATCCATTGTATGTGGATTTCCTCA  
 581 C-7: CAAATCCTAATCCAATATCAAATAGTATCC  
 613 C-7: AATAAATCTTTTTCTTGAATATCC  
 09/613B C-7: CGCGCACAGAAGGGCGAGAGACGA  
 640 C7: TTGTGGACAAATCACACAATTG  
 645 C7: GTGATTGTCCACAATATTTTAT

### *Pfdhps* extension primer

436: TTATAGATATAGGTGGAGAATCC  
 437: TTGGATTAGGTATAACAAAAGGA  
 540: AGGAAATCCACATACAATGGAT  
 581: GGATACTATTTGATATTGGATTAGGATTG  
 613: GGATATTCAAGAAAAAGATTTATT  
 09/613B: TCGTCTCTCGCCCTTCTGTGCGCGATTTTGATCATTTCATGCAATGGG  
 640: CAATTGTGTGATTGTCCACAA  
 645: ATAAAAATATTGTGGACAAATCAC

***Pfdhps* sequence (Accession Number: Z30659)**

P8-1 for

P8 for

P8 rev

P8-1 rev

C-7 and extension primer *dhps* 436, *dhps* 437, *dhps* 540, *dhps* 581, *dhps* 613, *dhps* 640, *dhps* 645

SNP

```

01  TGATACCCGAATATAAGCATAATGTTTTAAATAATACCATCAGATGTTTATATAACAAAT
61  ATGTGAGTAGGATGAAAGAACAATATAATATAAATATTAAAGAAAATAATAAAGGATAT
121 ATGTATTAAAAGATAGAATTTCTATTTAAAAGAAAAACAAATATTGTTGGAATATTAA
181 ATGTTAATTATGATTCTTTTTCAGATGGAGGTATTTTGTGTAACCTAAACGTGCTGTTTC
241 AAAGAATGTTTGAAATGATAAATGAAGGTGCTAGTGTTATAGATATAGGTGGAGAATCCG
301 CTGCTCCTTTTGTATACCTAATCCAAAAATTAGTGAAAGAGATTTAGTAGTACCTGTAT
361 TACAATTATTTCAAAAAGAATGGAATGATATAAAAAATAAAATTGTTAAATGTGATGCGA
421 AACCAATTATAAGTATTGATACAATTAATAATGTTTTAAAGAATGTGTTGATAATG
481 ATTTAGTTGATATATTAAATGATATTAGTGCTTGTACAAATAATCCAGAAATTATAAAAT
541 TATTAACCAATTAATCTATAGTGTAGTTCTAATGCATAAAAGAGGAAATCCAC
601 ATACAATGGATAACTAACAAATTATGATAATCTAGTTTATGATATAAAAAATTATTTAG
661 AACAAAGATTAAATTTTCTTGATTAAATGGAATACCTCGTTATAGGATACTATTTGATA
721 TTGGATTAGGATTTGCGAAGAAACATGATCAATCTATTAACTCTTACAAAATATACATG
781 TATATGATGAGTATCCACTTTTTATTGGATATTCAAGAAAAAGATTTATTGCCATTGCA
841 TGAATGATCAAAATGTTGTAATAAATACACAACAAAAATTACATGATGAACAACAAATG
901 AAAATAAAAATATTGTGGACAAATCACACAATTGGATGTTTCAGATGAATTACATGAGGA
961 AAGACAAGGATCAACTTTTATATCAAAAAATATATGTGGTGTGTTAAAAAAAAAAAAA
1021 AATTCAAATGAGTATACAAAAGTAACAATTCTATATATGTTACATATAAAATATAAATAA
1081 TATATATTCATGTATATGTATTATGTATTTCTTTTCAGGTGGATTAGCAATTGCTTCCT
1141 ACAGCTATTATAAAAGGTAGATCTAATAAGAGTTTCATGACGTTTTAGAAACAAAATCGG
1201 TTTTGGATGTTTTAACAAAATAGACCAAGTGTAAATTACAAAAGGAAAGTGCAACATG
1261 TGATTAAAC

```

### 7.4.3 *Pfmdr1*

#### PCR I:

*Pfmdr1* primary PCR I primer (Size PCR product: 613 bp)

P1-1 for: TTAATGTTTACCTGCACAACATAGAAAATT  
P1-1 rev : CTCCACAATAACTTGCAACAGTTCTTA

*Pfmdr1* nested PCR I primer (Size PCR product: 526 bp)

P1 for: TGTATGTGCTGTATTATCAGGA  
P1 rev: CTCTTCTATAATGGACATGGTA

*Pfmdr1* I C-7 primer

MDR Neg2 C-7: AAAACTACAGCAATCGTTGGAGAAACAGGT

86 C-7: CATGTTCTTTAATATTACACCAA  
13/184 C-7: GGGAACGACACAGACAAGCCGGGG

*Pfmdr1* I extension primer

86: TTTGGTGTAATATTAAAGAACATG  
13/184: CCCCGGCTTGTCTGTGTCGTTCCCTGCCAGTTCCTTTTAGGTTTAT

*Pfmdr1* sequence I (Accession Number: from S53996)

P1-1 for  
P1 for  
P1 rev  
P1-1 rev

C-7 and extension primer *mdr* 86, *mdr* 184

SNP

```

01 ATGGGTAAAGAGCAGAAAGAGAAAAAGATGGTAACCTCAGTATCAAAGAAGAGGTTGAA
61 AAAGAGTTGAACAAAAAGAGTACCGCTGAATTATTTAGAAAAATAAAGAATGAGAAAATA
121 TCATTTTTTTTACCGTTTAAATGTTTACCTGCACAACATAGAAAATTATTATTATATCA
181 TTTGTATGTGCTGTATTATCAGGAGGAACATTACCTTTTTTTATATCTGTGTTTGGTGTA
241 ATATTAAAGAACATGATTATTTAGGTGATGATATTAATCCTATAATATTATCATTAGTATCT
301 ATAGGTTTAGTACAATTTATATTATCAATGATATCAAGTTATTGTATGGATGTAATTACA
361 TCAAAAAATATTAAAAAAGCTTTAAAGCTTGAATATTTAAGAAGTGTTTTTTATCAAGATGGA
421 CAATTTTCATGATAATAATCCTGGATCTAAATTAAGATCTGATTAGATTTTTATTTAGAA
481 CAAGTGAGTTCAGGAATTGGTACGAAATTTATAACAATTTTACATA TGCCAGTTCCTTT
541 TTAGGTTTATATATTGTTGTCATTAATAAAAAATGCACGTTTGACTTTATGTATTACTTGC
601 GTTTTTCCGTTAATTTATGTTTGTGGTGTCATATGTAATAAGAAAGTAAATTTAAATAAA
661 AAAACATCTTTGTTATATAATAACAA TACCATGTCCATTATAGAAGAGGCTTTAATGGGA
721 ATAAGAAGCTGTGCAAGTTATTGTGGAGAAAAGACTATATTAAACAAATTTAATTGTCC
781 GAAACTTTTTTATAGTAAATATATTTTAAAGCTAATTTTGTAGAAGCATTACATATAGGT
841 TTAATAAATGGTTTAATTTTAGTTTCTTATGCATTCGGTTTTTGGTATGGTACAAGAATT
901 ATTATAAATAGTCAACGAATCAATACCCCAATAATGATTTTAATGGTGCCTCAGTTATA
961 TCCATTTTATTAGTGTAATTTAGTATGTTTATGTTAACAATTATCTTACCAATATA
1021 ACAGAATATATGAAAGCTTTAGAAGCAACAAATAGTTTATATGAAATAATAAATCGAAAA

```

**PCR II:****Pfmdr1 primary PCR II primer (Size PCR product: 880 bp)**

P3-1 for: AATTTGATAGAAAAAGCTATTGATTATAA  
 P3-1 rev: TATTGGTAATGATTCGATAAAATTCATC

**Pfmdr1 nested PCR II primer (Size PCR product: 799 bp)**

P3 for: GAATTATTGTAAATGCAGCTTTA  
 P3 rev: GCAGCAAACCTTACTAACACG

**Pfmdr1 II C-7 primer**

1034 C-7: GAATCCCCATAAAGCTGCATTTACAAT  
 1042 C-7: ATAGTTTTGCCTATTGGTTTGGATCCTTCT  
 1246 C-7: TCTTAAGTTATAATCACATATATTA

**Pfmdr1 II extension primer**

1034: ATTGTAAATGCAGCTTTATGGGGATT  
 1042: AGAAGGATCCAAACCAATAGGCAAACTAT  
 1246: TAATATATGTGATTATAACTTAAGA

**Pfmdr1 sequence II (Accession Number: from S53996)**

P3-1 for  
 P3 for  
 P3 rev  
 P3-1 rev

C-7 and extension primer *mdr* 1034, *mdr* 1042, *mdr* 1246

**SNP**

```

2701 AAAACGGGTTTAGTAAATAATATTGTTATTTCTCTCATTTCATAATGCTCTTCTGGTT
2761 AGCATGGTTATGTCCTTTTATTTTGTCCAATTGTTGCAGCTGTATTAACTTTTATATAT
2821 TTTATTAATATGCGTGTATTTGCTGTAAGAGCTAGATTAAACAAAAGTAAAGAAATTGAG
2881 AAAAAAGAAAATATGTCAAGCGGAGTTTTTGCATTTAGTTCAGATGATGAAATGTTTAAA
2941 GATCCAAGTTTTTAAATACAGGAAGCATTTTATAATATGCATACTGTTATTAATTATGGT
3001 TTAGAAGATTATTTCTGTAAATTTGATAGAAAAAGCTATTGATTATAAAAAATAAAGACAA
3061 AAAAGAAGAATTATTGTAAATGCAGCTTTATGGGGATTCAAGTCAAAGCGCTCAATTATTT
3121 ATTATAGTTTTGCCTATTGGTTTGGATCCTTCTTAATTAAAGAGGTACTATATTAGTT
3181 GATGACTTTATGAAATCCTTATTTACTTTTATATTTACTGGTAGTTATGCTGGAATAATTA
3241 ATGTCCTTAAAGGAGATTTCAGAAAATGCAAAATTATCATTGAGAAATATTATCCATTA
3301 ATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGAATAAGAATAAATAAAAAAT
3361 TTAATAAAAGGTAAAGTTGATATTAAAGATGTAAATTTCCGTTATATTTCAAGACCAAAT
3421 GTACCTATTTATAAAAAATTTATCTTTTACATGTGATAGTAAAAAACTACAGCAATCGTT
3481 GGAGAAACAGGTAGTGGAAAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTG
3541 AAAAAATGATCACATTATATTAAAAAATGATATGACAAATTTCAAGATTATCAAAAAAT
3601 AATAATAATTCATTGGTTTTAAAAAATGTAATGAATTTTCAAACCAATCTGGATCTGCA
3661 GAAGATTATACGTATTTAATAATAATGGAGAAATATTATTAGATGATATTAATATATGT
3721 GATTATAACTTAAGACATCTTAGAACTTATTTCAATAGTTAGTCAAGAACCCATGTTA
3781 TTTAATATGTCCATATATGAAAATATCAAATTTGGAAGAGAAGATGCAAATTTGAAGATC
3841 GTTAAA CGTGTAGTAAGTTTGCCTGCTATAGATGAATTTATCGAATCATTACCAAATAAA
3901 TATGATACAAATGTTGGACCATATGGTAAAAGCTTATCAGGTGGACAAAAACAGAGAATA

```

## 7.4.4 *Pfcr*

### PCR I:

*Pfcr* primary PCR I primer (Size PCR product: 280 bp)

P10-1 for: TTGTCGACCTTAACAGATGGCTCAC  
P10-1 rev: AATTCCCTTTTTATTTCCAAATAAGGA

*Pfcr* nested PCR I primer (Size PCR product: 200 bp)

P10-for: CTTGCTTGGTAAATGTGCTC  
P10-rev: GAACATAATCATACAAATAAAGT

*Pfcr* I C-7 primer

CRT Neg 3 C-7: AAACCTATTTTTTAAAGAGATTAAGGATAA  
01/72 C-7\*: ACGCCGGAACGCCGGAACGCCGGA  
75B1 C-7\*: AATTACACATACACTTAAATAAATAACTTAA  
02/74 C-7\*: ACGGGGCAACGGGGCAACGGGGCA  
76 C-7: AATTTTGCTAAAAGAACTTTAAACAAA  
97 C-7: TGAGTTTCGGATGTTACAAAAC

*Pfcr* I extension primer

01/72\*: TCCGGCGTTCCGGCGTTCCGGCGTTTTTAAGTATTATTTATTTAAGTGTA  
75B1\*: TTAAGTATTATTTATTTAAGTGTATGTGTAATT  
02/74\*: TGCCCCGTTGCCCCGTTGCCCCGTTATTATTTATTTAAGTGTATGTGTAAT  
76: TTTGTTAAAGTTCTTTTAGCAAAAATT  
97: GTTTGTAAACATCCGAAACTCA

(\* currently in test stage)

*Pfcr* sequence I (Accession Number: from AF030694)

P10-1 for

P10 for

P10 rev

P10-1 rev

C-7 and extension primer crt 72, crt 74, crt 75, crt 76, crt 97

SNP

```

01 AAATTCGCAAGTAAAAAATAATCAAAAAAATCAAGCAAAAATGACGAGCGTTATAGA
61 GAATTAGATAATTTAGTACAAGAAGGAAGTAAGTATCCAAAAATGGAAATATTGAATGAT
121 ATAAATGAATAGATAAAATCAACCTATTGGATATATATATATATATATATATATATA
181 TATGTATACCCATATGTATTAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCC
241 CTTGTCGACCTTAACAGATGGCTCACGTTTAGGTGGAGGTTCTTGTCTTGGTAAATGTGC
301 TCATGTGTTTAACTTATTTTTTAAAGAGATTAAGGATAATATTTTATTTATATTTTAAG
361 TATTATTTATTTAAGTGTATGTGTAATTGAAAACAATTTTGTCTAAAAGAACTTTAAACAA
421 AATTGGTAACTATAGTTTGTAAACATCCGAAACTCACACTTTATTTGTATGATTATGTT
481 CTTTATTGTTTATTCCTTATTTGGAAATAAAAAGGGAAATTCAAAAGTAAGATAAATCAA
541 TATATTAATATGATGGATTTATAAGAGAATCTATTCCACCTACCAATATAAAACATTACA
601 CATATATATATATATATATATATATATATATATATATATATATATATATATATATATA
661 TTTATATTTATTTCTTATGACCTTTTATAGGAACGACACCGAAGCTTTAATTTACAATTTT
721 TTGCTATATCCATGTAGATGCCTGTTTCAGTCATTTTGGCCTTCATAGGTCTTACAAGAA
781 CTACTGGAAATATCCAATCATTTGTTCTTCAATTAAGTATTCCTATTAATATGTTCTTCT
841 GCTTTTAAATATTAAGATATAGGTAAGTATACTATTTTAAATTACAAAAAATATATATA
901 AAAAAAAAAAAAAAAAAATATAAATATATATATATATATATATATATATATATATATA

```

*Pfcrt* primary PCR II primer (Size PCR product: 605 bp)

*Pfcrt* nested PCR II primer (Size PCR product: 548 bp)

*Pfcrt* II C-7 primer

*Pfcrt* II extension primer

***Pfcrt* sequence II (Accession Number: from AF030694)**

P18.1 for  
P18 for  
P18 rev  
P18.1 rev

C-7 and extension primer *crt* 152, *crt* 163

SNP

421 AATTGGTAACTATAGTTTTGTAACATCCGAAACTCACA**ACTTTATTTGTATGATTATGTT**  
481 **CTTTATTGTTTATTCCTTTATTTGGAAATAAAAAGGGAAATT**CAAAAGTAAGATAAAATCAA  
541 TATATTTAAATGATGGATTATAAGAGAATCTATTCCACCTACCAATATAAAACATTACA  
601 CATATATATATATATATATATATATATATATATGATGTATGTTGATTAAATTTGTTTATATA  
661 TTTATATTATTTCTTATGACCTTTTTAGGAACGACACCGAAGCTTTAATTTACAATTTT  
721 TTGCTATATCCATGTTAGATGCCTGTTTCAGTCATTTTGG**CCTTCATAGGTCTTACAAGAA**  
781 **CTA**CTGGAAAT**ATCCAATCATTTGTTCTTCAATTAA****GT**ATTCCATTAAATATGTTCTTCT  
841 GCTTTTTAATATTAAGATATAGGTAAGTATACTATTTTAAATTACAAAAAAAAAAAAAAAA  
901 AAAAAAAAAAAAAAAAAATATAAAATATATATATATATATATATATATATATATATATATA  
961 TATATTATATATATTATTATATATATTTATTTATTTATTTATTTATTTATTTATTTATTTA  
1021 **CTCCTTTTATAGATATCACTTAACAATTATCTCGGAGCAGTTA**TATTGTTGTAAACAATA



*Pfcrt* primary PCR III primer (Size PCR product: 360 bp)

*Pfcr*t nested PCR III primer (Size PCR product: 304 bp)

Pfcrt III C-7 Primer

*Pfcrt* III extension primer

220: TTCTATCATATTTAATCTTGTCTTAATTAGT

***Pfcrt* sequence III (Accession Number: from AF030694)**

P11-1 for  
P11 for  
P11 rev  
P11-1 rev

C-7 and extension primer *crt* 220,  
SNP

961 TATATTTATATATATTTATTTATATATTTATTTATTTATTTATTTATATTTATTTATTTA

1021 CTCCTTTTTCAGATATCACTTAACAATTATCTCGGAGCAGTTATTTATTGTTGTAACAATA

1081 GCTCTTGTAGAAATGAAATTATCTTTTGAAACACAAGAAGAAAATCTATCATATTTAAT

1141 CTGTGCTTAATTAGTTCCTTAATTGTAAGAAAACAAATATATAAATAAATAAATATATA

1201 TATATATATATATATATATATATATTTGTAATTTTAAATATATATTAACACTTAACTTTG

1261 TTTTATTATATTAATTTATATATTTCTTATCATTTTTTTTTTTTTTCTCTCTTTTTTT

1321 TTTAGCCTGTATGCTTTTCAACATGACAGGGAATAGTTTTTAAAAATATAAGATTG

1381 ACATTTTAAGATTAAATGTAAGAAGAAATATATAATAATAATAATATATATATATATATA

**PCR IV:****Pfcrt primary PCR IV primer** (Size PCR product: 697 bp)

P16-1 for: TCTGTTATTTTTATTCTTATAGGCTAT  
 P16-1 rev: CTTGTATGTATCAACGTTTTTCATCC

**Pfcrt nested PCR IV primer** (Size PCR product: 630 bp)

P16-for: CTTTTCCAATTGTTCACTTCTTG  
 P16-rev: TCTTACATAGCTGGTTATTAAAT

**Pfcrt IV C-7 primer**

271 C-7: TTTTAAAAATGGAAGGGTGTATA  
 326 C-7: AAAGAAGGAAAACAATGCGAAGGTTT  
 326B C-7: CATTTGTGATAATTTAATAACCAGCTATGT

**Pfcrt IV extension primer**

271: TATACACCCTTCCATTTTTAAAA  
 326: AAACCTTCGCATTGTTTCCTTCTTT  
 326B: ACATAGCTGGTTATTAAATTATCACAAATG

**Pfcrt sequence IV (Accession Number: from AF030694)**

P16-1for  
 P16 for  
 P16 rev  
 P16-1 rev

C-7 and extension primer crt 271, crt 326, crt 326B

SNP

```

1441 ATATTTCTTTTTTACCACTTTTTTTTTTTTTTTATTCCTATAACGCATTATAATTATTTCT
1501 GTTATTTTTATTCTTATAGGCTATGGTATCCTTTTCCAATTGTTCACTTCTTGTCTTA
1561 TATTACCTGTATACACCCTTCCATTTTTAAAACAACGTAAGAATTAATTAGGAAAGAAAA
1621 AATAAATAAATGAATGTGCCCATATATATATATATATATATATATATATATATATATGTA
1681 TGTATAATTTTCCCTTTTTAGTTCATTTACCATATAATGAAATATGGACAAATATAAAAA
1741 ATGGTTTCGCATGTTTATTCTTGGGAAGAAACACAGTCGTAGAGGTAAGGATTTTC
1801 ATTATATATTTAAAAATTACTACTTATTATGTTAATAAAAAAATATGTTTTTAAATGTTCA
1861 ATTTGTTTTATTTAATTATTTTTTTTTTTTTTTGTTTGTTTCCTCTTCAGAATTGTGGTC
1921 TTGGTATGGCTAAGTTATGTGATGATTGTGACGGAGCATGGGTAAGAAGCTTATAATAAA
1981 ATTTCAAAATTATAAGAGACATTTATATATATTTTAAACAATAATAATTAAATAAAACAAT
2041 ATTATATATATTATATATATATTATTATTTTATTATTATTTTTTTTTTTTTTTTTTTAGAA
2101 AACTTCGCATTGTTTCCTTCTTTAGCATTTGTGATAATTTAATAACCAGCTATGTAAG
2161 AATAAAAAGGATGAAAACGTTGATACATACAAG

```

**AGGAAATAAATATGGGAATGTTTAAT**

2221 TGAATTAAGATATATATATATATATATATATATATATATGTACCATATAATTTTCATT  
2281 TTCTATCTTTTTTATAGATTATCGACAAATTTTCT**ACCATGACATATACTATTGTTAGT**  
2341 GTATACAAGGTCACGCA**ACAGCAATTGCTTATTACTTTAAATTCTTAGCC**GTAAGAATTA  
2401 AAAAGATATAAAATATAAAATATATATGTGAATATATGATATATATATATATTTTT  
2461 TATATGTAATGTTTTTTTTTTTTTTTTCACAATATACATTTAAATGTTTATGATGGTACAA  
2521 CGTATCATATTTTATAAATAATTTTATGCATTCATGTATATTATTTTA**CTTTTTAAATTT**  
2581 ATAGGGTGATGTTGTAA**T**AGAACCAAGATATTAGATTTCGTAACCTTGGTAAGTGTGAA  
2641 ATTAAAAAATGAATTTTTTTTTTTTTTTTTTTTATGAACAAAATAATGTGTATATAA  
2701 TATGTGTGAATATCAAATGCTTGCTTGTTCGTTATAAATATTATTTTTTTTTTTTTTT  
2761 TTACAGTTT**GGCTACCTATTGTTCTATAA**TTT**ACCGTGTAGGAAATATTATCTTAGAA**  
2821 AGTAATACAAAAATAAGATAAAAAAATATAATATATAAAATATGTATATTGTTCTTATAT  
2881 ATTTTGTTCATATATATATATATATATATATATATTTTTATATTTCATCTGCTTTTTA  
2941 TTCTATTGTTATAATTTATCATAAAATTTTTTTTAAATTTGTTTACATTAGGAAAAAAAAAT  
3001 GAGAAATGAAGAAAAATGAAGATTCCGAAGGAGAAATTAACCAACGTCGATTCAATTATTAC  
3061 ACAATAA

## 7.4.5 *PfATPase6*

### *PfATPase6* primary PCR primer (Size PCR product: 896 bp)

P17-1 for                    AATATTGTTATTCAGAATATGATTATAA  
P17-1 rev                    TGGATCAATAATACCTAATCCACCTA

### *PfATPase6* nested PCR primer (Size PCR product: 798 bp)

P17 for                    AGCAAATATTTTCTGTAACGATAATA  
P17 rev                    TGTCTAATTTATAATAATCATCTGT

### *PfATPase6* C-7 primer

538 C-7:                    ACCGAATTAGCTTTATTACATT  
574 C-7:                    GTACAGGTGTTGTATTTTTTCA  
623 C-7:                    CCTGAGCTGTAGTATAATTAGAATGGTT  
683 C-7:                    TTTCTCCAAGAAGAAATACATTCA  
769 C-7:                    TTAATTTTTTATAAGCAAAGCTAAGT  
769B C-7                    TAGTAAAGATTTAAATATTAAGAATACAG

### *PfATPase6* extension primer

538:                    AAATGTAATAAAGCTAATTCGGT  
574:                    TGAAAAAATAACAACCTGTAC  
623:                    AACCATTCTAATTATACTACAGCTCAGG  
683:                    TGAATGTATTTCTTCTTGGAGAAA  
769:                    ACTTAGCTTTGCTTATAAAAAATTAA  
769B                    CTGTATTCTTAATATTTAAATCTTACTA

P17-1 for  
P17 for  
P17 rev  
P17-1 rev

SNP

1 AATGTGTTTATTTTATTATT  
61 TAATTTATTTTCGTTGAACCTATTATATCCTTTGTCATTTCGTGAAATTATTTATTATTATAC  
121 ATAATATTTTGTTTTGTATATAAAGAATGGAAGAGGTTATTAGAATGCTCATACATAC  
181 ATGTTGAGGATGTACTAAAAATTTTGGATGTAACAAGATAATGGTTTTAAAGAATGAGG  
241 AATTGGATGATAGAAGATTAATAATGTTTGAATGAATTAGAAGTAGAAAAGAAGAAAA  
301 GTATTTTGAATTGATATTAATCAATTTGATGATTTATTAGTAAAGATATTATTAGT  
361 CTGCATTCATTAGTTTTCTGTTAACTTTATTAGATATGAAACATAAAAAAATAAATAT  
421 GTGATTTTATTGAACCATTAGTTATAGTATTAATATTAATATTAAATGCTGCCGTAGGTG  
481 TATGGCAAAGATGTAATGCTGAAAAATCTTTAGAAGCTTTAAAGAATTACAACCTACC  
541 AAGCTAAAGTATTACGAGATGGGAAGTGGGAAATTATTGATAGTAAATATTTATATGTTG  
601 GTGATATTATTGAATTGAGTGTGGTAATAAACTCCCGCTGATGCAAGAATAATTAANA  
661 TATATTTCAACAAGTTTAAAAAGTTGAACAGAGTATGTTAACAGGAAATCCTGTTCAAGTT  
721 ACAAAATATGCTGAAAAAATGGAAGATAGTTATAAAAAATTTGGAATACAGTTGAAAAAAA  
781 ATATTTTATTTTTCATCTACCGCTATTGTATGTGGTAGATGTATAGCTGTTGTAATCAACA  
841 TAGGTATGAAGACTGAAATAGGTCATATTCAGCATGCTGTTATAGAATCAAATAGTGAAG  
901 ATACTCAAACACCTTTACAAATAAAAAATCGATTTATTTGGTCAACAATTATCAAANAATCA  
961 TTTTGTAAATATGTGTAACGTGATTGATTATTAATTTTAAACATTTCTCAGATCCAATTT  
1021 ATGGTTCACTTTTATATGTTTGTATATTATTTTAAATTTAGTGTGCTTTAGCTGTTG  
1081 CTGCTATACCGAAGGATTGCCAGCATCTATAACAACATTTGTTAGCTTTAGGACAAGAA  
1141 GAATGGTAAAAAANAATGCTATAGTAAGAAAAATTACAAAGTGTGAGACGTTAGGATGTA  
1201 CAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCG  
1261 TGTTTCATTTGTTTAGAGAATCTGATTCCTTAACAGAATACCAACTATGTCAAAAGGGG  
1321 ATACCTATTACTTTTATGAAAGTTCAAACCTTAACAAATGATATATATGCAGGTGAATCAT  
1381 CTTTTTTTTAATAATTAAGATGAAGGAAATGTTGAAGCTTTAACGGATGATGGAGAA  
1441 AAGGATCAATTGATGAAGCTGATCCATATAGTGATTATTTTTCTAGTGATAGTAAAGAA  
1501 TGAAAAATGATTTAAACAACAACAATAATAATAATAATAGTAGTAGGAGTGGTGCTA  
1561 AGAGGAATATTCCTTTAAAGAAATGAAATCAAATGAAATACAATAATAAGTAGAGGTA  
1621 GTAAAAATATTAGAAGATAAAATTAATAAATATTGTTATTCAGAATATGATTATAATTTT  
1681 ATATGTGTTTAGTAAATGTAAATGAAGCAATATTTCTGTACAGATAATAGTCAAATAG  
1741 TAAAAAATTTTGAGACAGTACCGAATTAGCTTTATTACATTTTGTACATAATTTTGATA  
1801 TATTACCAACATTTCTAAAAATAATAAATGCCAGCAGAAATGAAAAAATACACAC  
1861 CTGTACATCATCAAATGAAGGATAAATCACCAAGGGATACACAAATTTCTTAGTT  
1921 CAAAAATGATAACAGTCATATTACCAGTACATTGAATGAAATGATAAGAATTTAAAGA  
1981 ATGCTAACCATTTCTAATTATACTACAGCTCAGGCAACAACAAATGGATATGAAGCTATAG  
2041 GAGAAAAATACATTTGAGCATGGCACAAGTTTGAANAATGTTTCCACTCAAAATTGGGTA  
2101 ATAAAAATAAATACCACATCAACACATAATAATAAACAACAATAATAATAATAGTAATA  
2161 GTGTTCCAGTGAATGTATTTCTTTGGAGAAATGAATGTAAACAAATAAAAAATTATG  
2221 AATTCATAGAGAAAGGAACTTATAGTGTATTGTTGAAATAAAAAAAAAAAGAAATAA  
2281 TATTGTATTGTAAGGTGCACCTGAGAATAATAAAAAATTTGTAATATTATTTAACGA  
2341 AAAATGATATACGTCCATTAAATGAAACTTTAAAAAATGAAATTCATAATAAGATTCAA  
2401 ATATGGGAAAAAGAGCATTAAAGAACCTTAGCTTTGCTTATAAAAAATTAACTAGTAAAG  
2461 ATTTAAATATTAAAGAAATACAGATGATTATTATAAATTAGAACAAGATTTAATTTATTAG  
2521 GTGGATTAGGTATTATTGATCCACCACGTAATATGTAGGAAGAGCAATTAGCTATTGCC  
2581 ATATGGCTGGTATACGTGATTATTATGATTACAGGTGATAAATTAATACGGCCAGACTA  
2641 TAGCTAAAGAAATTAATATATTAATAAATAAAGGAAGATGATGAAAGGATAATTATA  
2701 CAAATAATAAAAAATACACAAATATGTTGTTATAATGGAAGAGAATTTGAAGATTTTTCAT  
2761 TAGAAAAGCAAAAACATATTTAAAAAATACACCAAGAATTTGTTTCTGTAGAACTGAAC  
2821 CTAACATAAAAAACAATAAGTAAAAGTATTAAAAGACTTAGGAGAAACAGTTGCTATGA  
2881 CAGGTGATGGTGAAATGATGCCACGACCTGAAATCAGCTGACATAGGAATAGCTATGG  
2941 GTATTAATGGAACGGAGGTAGCTAAAGAAGCATCAGATATTGTTTAGCTGATGATAATT  
3001 TTAATACATAGTTGAAGCAATTAAGAAGGAAGATGTATATAATAAATATGAAGCA

## 7.4.6 Position controls

**BIN CONTROL Cy3:** TAATTATGAAAATAAAGGAG

**BIN CONTROL Cy5:** TAATTATGAAAATAAAGGAG

## 7.4.5 Flexible primers

### NOTE:

- Regular: C-7 flexi tags free
- *Italic*: flexi extension tags free
- **Bold**: flexi C-7 tags in use!!!
- *Italic underlined*: flexi extension tags in use!!!

### • Flexible C-7 primers

01 C-7:	<b>ACGCCGGAACGCCGGAACGCCGGA</b>	( <i>crt</i> 72)
02 C-7:	<b>ACGGGGCAACGGGGCAACGGGGCA</b>	( <i>crt</i> 74)
03 C-7:	CAAGGCGCCAAGGCGCCAAGGCGC	
04 C-7:	CAGCGGCACAGCGGCACAGCGGCA	
05 C-7:	CCACACGGCCACACGGCCACACGG	
06 C-7:	<b>GGGAGAGCGCAGCAGGCAACAGAG</b>	( <i>dhfr</i> 164)
07 C-7:	<b>GACCGCCACCAAGAACAGCACC</b>	( <i>dhfr</i> 164B)
08 C-7:	GCGCCAACGCAGACCGGAAGACCA	
09 C-7:	<b>CGCGCACAGAAGGGCGAGAGACGA</b>	( <i>dhps</i> 613B)
10 C-7:	<b>GCAGGGAAGCGGGAGCGAAACAGC</b>	( <i>dhfr</i> 108)
11 C-7:	<b>AAAACGGGGCACAGCGCGCGGAA</b>	( <i>dhfr</i> 108B)
12 C-7:	CCCCGAGAACGCCCGAAGCACAAG	
13 C-7:	<b>GGGAACGACACAGACAAGCCGGGG</b>	( <i>mdr1</i> 184)
14 C-7:	GGCGGGAACCGAAGACAGGGGAAG	
15 C-7:	GACAACGGGCAGCGACCGGACCAA	
16 C-7:	CCCAAAGCCCGCAACCCGACCAAC	
17 C-7:	CAGGAACCAAGCCAGCCAGAGGCC	
18 C-7:	ACACCACAGGACACACGCCCCAGG	

### • Flexible extension primers

01/72*:	<u>TCCGGCGTTCCGGCGTTCCGGCGTTTTTAAGTATTATTTATTTAAGTGTA</u>
02/74*:	<u>TGCCCCGTTGCCCGTTGCCCGTTATTATTTATTTAAGTGTATGTGTAAT</u>
03/:	<u>GTTCCGCGGTTCCGCGGTTCCGCG</u>
04/:	<u>GTCGCCGTGTCGCCGTGTCGCCGT</u>
05/:	<u>GGTGTGCCGTGTGCCGTGTGCC</u>
06/164:	<u>CTCTGTGCCTGCTGCGCTCTCCCGGAAATTAAATTACTATAAAATGTTTTATT</u>
07/164B:	<u>CCGGTGCTGTTCTTGGTGGCGGTCTTCTTGATAAACAACGGAACCTCCTA</u>
08/:	<u>TGGTCTTCCGGTCTGCGTTGGCGC</u>
09/613B:	<u>TCGICTCTCGCCCTTCTGTGCGCGATTTTGATCATTCAATGGA</u>
10/108:	<u>GCTGTTTCGCTCCCGCTTCCCTGCCAAAATGTTGTAGTTATGGGAAGAACAA</u>
11/108B:	<u>TTCCGCCGCGCTGTGCCCGTTTTAAAGGTTTAAATTTTTTGGAAATGCTTTCCAG</u>
12/:	<u>CTTGTGCTTCGGGCTTCTCGGGG</u>
13/184:	<u>CCCCGCTTGTCTGTGCTGTTCCCTGCCAGTTCCTTTTTAGGTTTAT</u>
14/:	<u>CTTCCCTGTCTTCGGTTCCCGCC</u>
15/:	<u>TTGGTCCGCTCGCTGCCCGTTGTC</u>
16/:	<u>GTTGGTCCGGTTGCGGGCTTTGGG</u>
17/:	<u>GGCCTCTGGCTGGCTTGGTTCTG</u>
18/:	<u>CCTGGGGCGTGTGCTGTGGTGT</u>

(\* currently in test stage)

## 7.5 Oligonucleotide array on the chip

Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!
Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!
Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!	Empty!

- galDesigner software: Template saved as *malaria.sti*

### 7.5b Oligonucleotide array on the chip

(Intermediate batch designed by Jutta, May-July 2006)

Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	Empty!	Empty!
Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	Empty!	Empty!
Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	Empty!	Empty!

- galDesigner software: Template saved as *genopole\_May06.sti*

For slide printing, 0.5 nl of a 50  $\mu$ M solution of the C-7 primers are used per spot (Prior to printing, the 500  $\mu$ M C7-primer stock solutions have to be diluted 1:10 in 180 mM phosphate buffer pH 8.0).

## 7.6 SNPs on the chip

<i>Pfdhps</i>	Polymorphisms			CHIP				
436	TCT→GCT/TTT	Ser→Ala/Phe	S→A/F	TCT→GCT	CII	S→A	Cy3=WT	Cy5=MUT1
437***	GCT→GGT	Ala→Gly	A→G	GCT→GGT	CI	A→G	Cy3=MUT	Cy5=WT
540	AAA→GAA	Lys→Glu	K→E	AAA→GAA	CI	K→E	Cy3=WT	Cy5=MUT
581	GCG→GGG	Ala→Gly	A→G	GCG→GGG	CI	A→G	Cy3=WT	Cy5=MUT
613	GCC→ACC	Ala→Thr	A→T	GCC→ACC	CI	A→T	Cy3=MutA	Cy5=WT or MutB
613B***	GCC→TCC	Ala→Ser	A→S	GCC→TCC	CII	A→S	Cy3=WT or MutA	Cy5=MutB
640***	ATT→TTT	Ile→Phe	I→F	ATT→TTT	CI	I→F	Cy3=MUT	Cy5=WT
645	CAC→CCC	His→Pro	H→P	CAC→CCC	CII	H→P	Cy3=MUT	Cy5=WT
<i>Pfdhfr</i>	Polymorphisms			CHIP				
16	GCA→GTA	Ala→Val	A→V	GCA→GTA	CI	A→V	Cy3=WT	Cy5=MUT
51	AAT→ATT	Asn→Ile	N→I	AAT→ATT	CI	N→I	Cy3=WT	Cy5=MUT
59***	TGT→CGT	Cys→Arg	C→R	TGT→CGT	CI	C→R	Cy3=WT	Cy5=MUT
108	AGC→AAC	Ser→Asn	S→N	AGC→AAC	CI	S→N	Cy3=MutA or MutB	Cy5=WT
108B***	AGC→ACC	Ser→Thr	S→T	AGC→ACC	CII	S→T	Cy3=WT or MutA	Cy5=MutB
164	ATA→TTA	Ile→Leu	I→L	ATA→TTA	CI	I→L	Cy3=WT	Cy5=MUT
164B***	ATA→TTA	Ile→Leu	I→L	ATA→TTA	CII	I→L	Cy3=WT	Cy5=MUT
<i>Pfmdr1</i>	Polymorphisms			CHIP				
86	AAT→TAT	Asn→Tyr	N→Y	AAT→TAT	CI	N→Y	Cy3=WT	Cy5=MUT
184	TAT→TTT	Tyr→Phe	Y→F	TAT→TTT	CI	Y→F	Cy3=WT	Cy5=MUT
1034	AGT→TGT	Ser→Cys	S→C	AGT→TGT	CI	S→C	Cy3=WT	Cy5=MUT
1042***	AAT→GAT	Asn→Asp	N→D	AAT→GAT	CI	N→D	Cy3=MUT	Cy5=WT
1246	GAT→TAT	Asp→Tyr	D→Y	GAT→TAT	CII	D→Y	Cy3=MUT	Cy5=WT
<i>Pfprt</i>	Polymorphisms			CHIP				
72	TGT→AGT	Cys→Ser	C→S	TGT→AGT	CI	C→S	Cy3=MUT	Cy5=WT
74	ATG→ATT	Met→Ile	M→I	ATG→ATT	CII	M→I	Cy3=MUT	Cy5=WT
75B1	AAT→GAT/GAA	Asn→Asp/Glu	N→D/E	AAT→GAT/GAA	CI	N→D or E	Cy3=WT	Cy5=MUT
76***	AAA→ACA	Lys→Thr	K→T	AAA→ACA	CII	K→T	Cy3=WT	Cy5=MUT
97	CAC→CAA	His→Gln	H→Q	CAC→CAA	CII	H→Q	Cy3=WT	Cy5=MUT
152	ACT→GCT	Thr→Ala	T→A	ACT→GCT	CI	T→A	Cy3=WT	Cy5=MUT
163	AGT→AGG	Ser→Arg	S→R	AGT→AGG	CII	S→R	Cy3=WT	Cy5=MUT
220	GCC→TCC	Ala→Ser	A→S	GCC→TCC	CII	A→S	Cy3=MUT	Cy5=WT
271	CAA→GAA	Gln→Glu	Q→E	CAA→GAA	CI	Q→E	Cy3=WT	Cy5=MUT
326	AAC→GAC	Asn→Asp	N→D	AAC→GAC	CI	N→D	Cy3=WT	Cy5=MUT1
326B***	AAC→AGC	Asn→Ser	N→S	AAC→AGC	CI	N→S	Cy3=MUT2	Cy5=WT
356	ATA→TTA	Ile→Leu	I→L	ATA→TTA	CI	I→L	Cy3=WT	Cy5=MUT1
356B***	ATA→ACA	Ile→Thr	I→T	ATA→ACA	CI	I→T	Cy3=WT	Cy5=MUT2
371	AGA→ATA	Arg→Ile	R→I	AGA→ATA	CII	R→I	Cy3=MUT	Cy5=WT
<i>PfATPase6</i>	Polymorphisms			CHIP				
538***	AGT→AGC	Ser→Arg	S→R	AGT→AGC	CI	S→R	Cy3=MUT	Cy5=WT
574	CAA→CCA	Gln→Pro	Q→P	CAA→CCA	CII	Q→P	Cy3=MUT	Cy5=WT
623	GCA→GAA	Ala→Glu	A→E	GCA→GAA	CII	A→E	Cy3=WT	Cy5=MUT
683	AAT→AAG	Asn→Lys	N→K	AAT→AAG	CII	N→K	Cy3=WT	Cy5=MUT
769	AGT→AAT	Ser→Asn	S→N	AGT→AAT	CI	S→N	Cy3=MUT	Cy5=WT
769B***	AGT→AAT	Ser→Asn	S→N	AGT→AAT	CI	S→N	Cy3=WT	Cy5=MUT

(\*\*\*) = extension on antisense strand)



## 7.7 PCR conditions for QIAGEN® *Taq* polymerase

### 7.7.1 Primary PCR mix

Reagents	1 reaction	14 reactions
H <sub>2</sub> O	33.25 µl	465.5 µl
10 x buffer (containing 15 mM MgCl <sub>2</sub> !)	5.0 µl	70.0 µl
dNTP mix (2mM)	5.0 µl	70.0 µl
MgCl <sub>2</sub> (25mM)	3.0 µl	42.0 µl
Primary PCR primer mix (10 µM each)	1.0 µl	14.0 µl
<i>Taq</i> Polymerase 5U/ µl	0.25 µl	3.5 µl
<b>Final Volume</b>	<b>47.5 µl</b>	<b>665.0 µl</b>
DNA	2.5 µl	
<b>Final Volume</b>	<b>50.0 µl</b>	

### 6.7.2 Primary PCR program

96 °C 180 Sec

96 °C 30 sec

52 °C 90 sec

72 °C 90 sec

20 cycles for clinical (symptomatic) samples

25 cycles for community (asymptomatic) samples

Hold at 4 °C

### 7.7.3 Nested PCR mix

Reagents	1 reaction	14 reactions
H <sub>2</sub> O	66.5 µl	931.0 µl
10 x buffer (containing 15 mM MgCl <sub>2</sub> !)	10.0 µl	140.0 µl
dNTP mix (2mM)	10.0 µl	140.0 µl
MgCl <sub>2</sub> (25mM)	6.0 µl	84.0 µl
Nested PCR primer mix (10 µM each)	2.0 µl	28.0 µl
<i>Taq</i> Polymerase 5U/ µl	0.5 µl	7.0 µl
<b>Final Volume</b>	<b>95.0 µl</b>	<b>1330.0 µl</b>
Primary PCR product	5.0 µl	
<b>Final Volume</b>	<b>100.0 µl</b>	

### 6.7.4 Nested PCR program

96 °C 180 sec

96 °C 30 sec

52 °C 90 sec

72 °C 90 sec

20 cycles for clinical (symptomatic) samples

25 cycles for community (asymptomatic) samples

Hold at 4 °C



## **Appendix IV**

### **Descriptive data tables**

## Appendix IV

SITE PROVINCE HEALTH FACILITY CATCHMENT AREA YEAR	2003 n=265	Karimui area Simbu Sigimaru health centre 6'000-7'000 2004 n=347	2005 n=359
TOTAL NUMBER OF INDIVIDUALS SURVEYED			
<b><u>Characteristics of study population</u></b>			
Sex (x/n (%))	F: 137/265 (51.70) M: 128/265 (48.30)	F: 192/347 (55.33) M: 155/347 (44.67)	F: 186/359 (51.81) M: 173/359 (48.19)
Age (mean (95% CI), yrs)	18.85 (16.98-20.71)	19.43 (17.72-21.15)	15.41 (14.01-16.81)
<b><u>Endemicity</u></b>			
Spleen rate all age groups (% (95% CI))	nd	12.79 (9.45-16.79)	21.43 (17.24-26.10)
Spleen rate 2-9 years (% (95% CI))	nd	26.85 (18.78-36.24)	35.82 (27.73-44.55)
Spleen rate adults (>20 years) (% (95% CI))	nd	4.96 (2.02-9.96)	6.25 (2.55-12.45)
<i>Pf</i> prevalence in children 2-9 years (x/n (% (95% CI))	13/93 (13.98, 7.66-22.72)	20/107 (18.69, 11.81-27.38)	44/138 (31.88, 24.21-40.35)
<b><u>Prevalence by microscopy</u></b>			
<i>Pf</i> prevalence (x/n, (%))	34/258 (13.18)	64/346 (18.50)	82/358 (22.91)
<i>Pv</i> prevalence (x/n, (%))	14/258 (5.43)	39/346 (11.27)	41/358 (11.45)
<i>Pm</i> prevalence (x/n, (%))	5/258 (1.94)	10/346 (2.89)	9/358 (2.51)
<i>Po</i> prevalence (x/n, (%))	0/258 (0.00)	0/346 (0.00)	0/358 (0.00)
Overall malaria prevalence (all species, (x/n, (%))	49/258 (18.99)	106/346 (30.64)	119/358 (33.24)
<b><u>Mixed infections</u></b>			
0=no infection (x/n, (%))	209/258 (81.01)	240/346 (69.36)	239/358 (66.76)
<i>Pf</i> single infection (x/n, (%))	31/258 (12.02)	57/346 (16.47)	71/358 (19.83)
<i>Pv</i> single infection (x/n, (%))	11/258 (4.26)	32/346 (9.25)	29/358 (8.10)
<i>Pm</i> single infection (x/n, (%))	4/258 (1.55)	10/346 (2.89)	6/358 (1.68)
<i>Pf</i> plus <i>Pv</i> double infection (x/n, (%))	2/258 (0.78)	7/346 (2.02)	10/358 (2.79)
<i>Pf</i> plus <i>Pm</i> double infection (x/n, (%))	0/258 (0.00)		1/358 (0.28)
<i>Pv</i> plus <i>Pm</i> double infection (x/n, (%))	0/258 (0.00)		2/358 (0.56)
<i>Pf</i> plus <i>Pv</i> plus <i>Pm</i> triple infection (x/n, (%))	1/258 (0.39)		
<i>Pf</i> density (geometric mean (range), per µl)	782 (0-43800)	357 (0-81600)	571 (0-138160)

## Appendix IV

SITE PROVINCE YEAR	Karimui area Simbu		
	2003 n=265	2004 n=347	2005 n=359
TOTAL NUMBER OF INDIVIDUALS SURVEYED			
<b><u>Prevalence by PCR</u></b>			
<i>Pf</i> prevalence by microscopy (x/n (%), (95% CI))	34/258 (13.18, 9.30-17.92)	64/346 (18.50, 14.54-22.97)	82/358 (22.91, 18.65-27.61)
<i>Pf</i> prevalence by <i>m</i> sp2 nPCR (x/n (%), (95% CI))	102/263 (38.78, 32.86-44.96)	71/347 (20.46, 16.34-25.09)	131/359 (36.49, 31.50-41.70)
Mean multiplicity of infection (MOI) (95% CI, range)	1.46 (1.32-1.60, 1-4)	1.59 (1.38-1.80, 1-4)	1.77 (1.62-1.92, 1-4)
<b><u>Pattern of MOI (<i>m</i>sp2 genotypes)</u></b>			
Single (x/n, (%))	65/102 (63.73)	44/71 (61.97)	61/131 (46.56)
Double (x/n, (%))	29/102 (28.43)	16/71 (22.54)	45/131 (34.35)
Triple (x/n, (%))	6/102 (5.88)	7/71 (9.86)	19/131 (14.50)
Quadruple (x/n, (%))	2/102 (1.96)	4/71 (5.63)	6/131 (4.58)
Quintuple (x/n, (%))			
Sextuple (x/n, (%))			
Prevalence 3D7 allele family (x/n, (%))	70/102 (68.63)	48/71 (67.60)	93/131 (70.99)
Prevalence FC27 allele family (x/n, (%))	57/102 (55.88)	42/71 (59.15)	91/131 (69.46)
<b><u>Symptoms</u></b>			
Temperature (mean (95% CI), °C)	36.06 (36.00-36.13)	36.09 (36.02-36.16)	36.42 (36.36-36.49)
Fever cases (temperature =37.5°C), x/n, (%), 95% CI)	3/264 (1.14, 0.23-3.28)	8/347 (2.31, 1.00-4.49)	16/359 (4.46, 2.57-7.14)
Hb (mean (95% CI), g/dl)	11.47 (11.19-11.76)	12.05 (11.83-12.27)	11.44 (11.22-11.66)
Anaemia (Hb <7.5 g/dl, x/n, (%), 95% CI)	12/263 (4.56, 2.38-7.83)	6/344 (1.74, 0.64-3.76)	10/359 (2.79, 1.34-5.06)

## Appendix IV

SITE PROVINCE YEAR	TOTAL NUMBER OF INDIVIDUALS SURVEYED	Karimui area		
		Simbu	2004	2005
		n=265	n=347	n=359
<b><i>Drug pressure</i></b>				
<b><i>Consumption at health facilities</i></b>				
Health book checked (x/n, (%))		8/265 (3.02%)	37/347 (10.66)	119/359 (33.15)
Antimalarial treatment courses in previous year (mean, (95%CI))		1.37 (0.12-2.63)	1.81 (1.22-2.40)	1.39 (1.17-1.60)
Antimalarial treatment courses in previous year (median, (range))		1 (1-5)	1 (0-6)	1 (0-4)
Combination therapy last course (x/n, (%))		8/8 (100%)	28/35 (80.00)	95/107 (88.79)
<b><i>Consumption outside health facilities</i></b>				
Consumption of drugs outside health facilities (x/n=y, (%))		28/265 (10.57)	3/347 (0.86)	0/359 (0.00)
Pharmacy (x/y, (%))		11/28 (39.28)	0/3 (0.00)	
Store (x/y, (%))		3/28 (10.71)	0/3 (0.00)	
Market (x/y, (%))		0/28 (0.00)	0/3 (0.00)	
Healer (x/y, (%))		0/28 (0.00)	0/3 (0.00)	
Relatives (x/y, (%))		0/28 (0.00)	0/3 (0.00)	
Other places (x/y, (%))		18/28 (64.28)	3/3 (100.00)	
Missionary		17/28 (60.71)	3/3 (100.00)	
Marasin meri				
Local aid post				
Home supply (staff health facility, friends)		1/28 (3.57)		
<b><i>Reported drugs consumed outside health facilities</i></b>				
Antimalarials (x/y, (%))		15/28 (83.33)	1/3 (33.33)	
Pain killers (e.g., Paracetamol, Aspirin, etc.) (x/y, (%))		14/28 (50.00)		
Unknown drugs (x/y, (%))		5/28 (17.86)	2/3 (66.67)	
Local herbs (x/y, (%))		1/28 (3.57)		
Antibiotics (x/y, (%))				

## Appendix IV

SITE PROVINCE HEALTH FACILITY CATCHMENT AREA YEAR	South Wosera East Sepik Kunjingini health centre (HC) 12'000-15'000 2003 n=317	2004 n=366	North Coast area Madang Mugil HC 15'000-20'000 2004 n=359
TOTAL NUMBER OF INDIVIDUALS SURVEYED			
<b><u>Characteristics of study population</u></b>			
Sex (x/n (%))	F: 159/317 (50.16) M: 158/317 (49.84)	F: 189/366 (51.64) M: 177/366 (48.36)	F: 182/359 (50.70) M: 177/359 (49.30)
Age (mean (95% CI), yrs)	20.68 (18.84-22.53)	21.33 (19.57-23.08)	20.06 (18.12-21.99)
<b><u>Endemicity</u></b>			
Spleen rate all age groups (% (95% CI))	7.46 (4.74-11.07)	7.44 (4.96-10.64)	25.36 (20.86-30.28)
Spleen rate 2-9 years (% (95% CI))	17.65 (10.23-27.43)	17.27 (10.73-25.65)	41.13 (32.37-50.32)
Spleen rate adults (>20 years) (% (95% CI))	0.00 (0.00-3.02)	0.67 (0.02-3.66)	10.96 (6.39-17.19)
<i>Pf</i> prevalence in children 2-9 years (x/n (% (95% CI))	21/98 (23.60, 15.24-33.78)	36/110 (32.73, 24.08-42.33)	46/128 (35.94, 27.65-44.89)
<b><u>Prevalence by microscopy</u></b>			
<i>Pf</i> prevalence (x/n, (%))	55/314 (17.52)	96/356 (26.97)	82/358 (22.91)
<i>Pv</i> prevalence (x/n, (%))	31/314 (9.87)	62/356 (17.42)	48/358 (13.41)
<i>Pm</i> prevalence (x/n, (%))	20/314 (6.37)	12/356 (3.37)	11/358 (3.07)
<i>Po</i> prevalence (x/n, (%))	0/314 (0.00)	0/356 (0.00)	0/358 (0.00)
Overall malaria prevalence (all species, (x/n, (%))	97/314 (30.89)	151/356 (42.42)	120/358 (33.52)
<b><u>Mixed infections</u></b>			
0=no infection (x/n, (%))	217/314 (69.11)	205/356 (57.58)	238/358 (66.48)
<i>Pf</i> single infection (x/n, (%))	47/314 (14.97)	78/356 (21.91)	63/358 (17.60)
<i>Pv</i> single infection (x/n, (%))	24/314 (7.64)	46/356 (12.92)	30/358 (8.38)
<i>Pm</i> single infection (x/n, (%))	17/314 (5.41)	8/356 (2.25)	7/358 (1.96)
<i>Pf</i> plus <i>Pv</i> double infection (x/n, (%))	6/314 (1.91)	15/356 (4.21)	16/358 (4.47)
<i>Pf</i> plus <i>Pm</i> double infection (x/n, (%))	2/314 (0.64)	3/356 (0.84)	2/358 (0.56)
<i>Pv</i> plus <i>Pm</i> double infection (x/n, (%))	1/314 (0.32)	1/356 (0.28)	1/358 (0.28)
<i>Pf</i> plus <i>Pv</i> plus <i>Pm</i> triple infection (x/n, (%))			1/358 (0.28)
<i>Pf</i> density (geometric mean (range), per µl)	461 (0-18800)	513 (0-153680)	1156 (0-65680)

## Appendix IV

SITE PROVINCE YEAR TOTAL NUMBER OF INDIVIDUALS SURVEYED	South Wosera East Sepik		North Coast area Madang	
	2003 n=317	2004 n=366	2004 n=359	
<b><u>Prevalence by PCR</u></b>				
<i>Pf</i> prevalence by microscopy (x/n (%), (95% CI))	55/314 (17.52, 13.48-22.18)	96/356 (26.97, 22.42-31.90)	82/358 (22.91, 18.65-27.61)	
<i>Pf</i> prevalence by <i>m</i> sp2 nPCR (x/n (%), (95% CI))	129/317 (40.69, 35.24-46.32)	147/366 (40.16, 35.10-45.38)	115/359 (32.03, 27.23-37.13)	
Mean multiplicity of infection (MOI) (95% CI, range)	1.77 (1.57-1.96, 1-6)	1.85 (1.68-2.02, 1-5)	1.54 (1.38-1.70, 1-5)	
<b><u>Pattern of MOI (<i>m</i>sp2 genotypes)</u></b>				
Single (x/n, (%))	71/129 (55.04)	73/147 (49.66)	73/115 (63.48)	
Double (x/n, (%))	32/129 (24.81)	36/147 (24.49)	27/115 (23.48)	
Triple (x/n, (%))	18/129 (13.95)	28/147 (19.05)	12/115 (10.43)	
Quadruple (x/n, (%))	2/129 (1.55)	7/147 (4.76)	1/115 (0.87)	
Quintuple (x/n, (%))	5/129 (3.88)	3/147 (2.04)	2/115 (1.74)	
Sextuple (x/n, (%))	1/129 (0.78)			
Prevalence 3D7 allele family (x/n, (%))	81/129 (62.79)	106/147 (72.11)	87/115 (75.65)	
Prevalence FC27 allele family (x/n, (%))	91/129 (70.45)	88/147 (59.86)	60/115 (52.17)	
<b><u>Symptoms</u></b>				
Temperature (mean (95% CI), °C)	36.55 (36.50-36.60)	36.52 (36.47-36.57)	36.40 (36.34-36.45)	
Fever cases (temperature =37.5°C), x/n, (%), 95% CI)	7/316 (2.22, 0.89-4.51)	8/266 (2.19, 0.89-4.02)	9/358 (2.51, 1.15-4.72)	
Hb (mean (95% CI), g/dl)	10.70 (10.51-10.89)	10.74 (10.59-10.89)	10.44 (10.26-10.62)	
Anaemia (Hb <7.5 g/dl, x/n, (%), 95% CI)	11/310 (3.55, 1.78-6.26)	30/363 (2.75, 1.33-5.01)	13/35 (3.63, 1.95-6.13)	



## Appendix IV

SITE PROVINCE YEAR	South Wosera East Sepik	North Coast area Madang	
2003 n=317	2004 n=366	2004 n=359	
TOTAL NUMBER OF INDIVIDUALS SURVEYED			
<i>Drug pressure</i>			
<i><u>Consumption at health facilities</u></i>			
Health book checked (x/n, (%))	241/317 (76.03)	277/366 (75.68)	292/359 (81.34)
Antimalarial treatment courses in previous year (mean, (95%CI))	1.20 (1.02-1.37)	1.47 (1.26-1.68)	1.37 (1.16-1.58)
Antimalarial treatment courses in previous year (median, (range))	1 (0-7)	1 (0-9)	1 (0-11)
Combination therapy last course (x/n, (%))	170/215 (79.07)	235/257 (91.44)	201/267 (75.28)
<i><u>Consumption outside health facilities</u></i>			
Consumption of drugs outside health facilities (x/n=y, (%))	36/317 (11.36)	38/366 (10.38)	81/359 (22.56)
Pharmacy (x/y, (%))	0/36 (0.00)	8/38 (21.05)	22/81 (27.16)
Store (x/y, (%))	1/36 (2.78)	9/38 (23.68)	7/81 (8.64)
Market (x/y, (%))	0/36 (0.00)	0/38 (0.00)	0/81 (0.00)
Healer (x/y, (%))	0/36 (0.00)	2/38 (5.26)	4/81 (4.94)
Relatives (x/y, (%))	8/36 (22.22)	6/38 (15.79)	27/81 (33.33)
Other places (x/y, (%))	31/36 (86.11)	25/38 (65.79)	36/81 (44.44)
Missionary			
Marasin meri	31/36 (86.11)	24/38 (63.16)	
Local aid post			19/81 (23.46)
Home supply (staff health facility, friends)		1/38 (2.63)	15/81 (18.52)
<i><u>Reported drugs consumed outside health facilities</u></i>			
Antimalarials (x/y, (%))	35/36 (97.22)	36/38 (94.74)	65/81 (80.25)
Pain killers (e.g., Paracetamol, Aspirin, etc.) (x/y, (%))	1/36 (2.78)	6/38 (15.79)	34/81 (41.97)
Unknown drugs (x/y, (%))	1/36 (2.78)	1/38 (2.63)	1/81 (1.23)
Local herbs (x/y, (%))			4/81 (4.94)
Antibiotics (x/y, (%))			9/81 (11.11)



## Curriculum vitae

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2003 - 2006: PhD student (Epidemiology) at the University of Basel (Swiss Tropical Institute), Switzerland; "Drug resistant malaria in Papua New Guinea and molecular monitoring of parasite resistance"; Supervision: PD Dr. B. Genton & Prof. H.-P. Beck  
1998 – 2002: Masters studies at the London School of Hygiene and Tropical Medicine (LSHTM), University of London, UK, External Program; MSc thesis: "Standardisation of an PCR-RFLP-based genotyping assay for the diagnosis of *Leishmania spp.*"; Supervision: Prof. H.-P. Beck; Masters of Science Diploma in Control of Infectious Diseases  
1988 – 1990: Education to Academic Technical Assistant (ATA) at the Swiss Tropical Institute, University of Basel, Switzerland; Supervision: Dr. H.-P. Marti & Prof. N. Weiss; ATA diploma thesis: "Zymodeme analysis of *Entamoeba histolytica*: Evaluation of its application as a routine diagnostic tool"; Bachelor of Science Diploma in Medical Parasitology  
1985 – 1988: 1st and 2nd year's course for medical students at the Faculty of Medicine of the University of Basel, Switzerland; advanced GEC A-level certificates  
1978 – 1985: High-school in Sursee, Switzerland; GEC A-level certificate (Typus B)

### *Postgraduate and training courses:*

2005: Surveillance of Communicable Diseases (Module in the tropEd Masters Programme in International Health), Basel, Switzerland  
2000: Postgraduate Diploma in Infectious Diseases at the London School of Hygiene and Tropical Medicine (LSHTM), London, UK  
1997: *In situ* Methods for Nucleic Acid Demonstration in Cells (R. R. Friis PhD, Faculty of Medicine, Department of Clinical Research), University of Berne, Switzerland  
1991: Introductory course in molecular parasitology (Dr. D. Dobbelaere, Institute for Livestock Pathology, Department of Parasitology), University of Berne, Switzerland

### *Employment:*

2003 - 2006: PhD student at the Swiss Tropical Institute (STI) in Basel, Switzerland, Department of Molecular Parasitology & Infection Biology and Department of Public Health & Epidemiology

- 1999 - 2003: Part-time employment as research assistant at the Swiss Tropical Institute (STI) in Basel, Switzerland, Department of Diagnostics and Department of Molecular Parasitology & Infection Biology
- 1997 - 1999: Permanent employment as research assistant at the University Hospitals of Basel, Switzerland, Department of Research (ZLF), Laboratory of Immunonephrology
- 1990 - 1997: Permanent employment as research assistant at the Swiss Tropical Institute (STI) in Basel, Switzerland, Department of Diagnostics and Department of Immunology
- 1985 - 1989: Part-time employment as an auxiliary nurse at the District Hospital of Sursee, Switzerland
- 1984 - 1986: Part-time employment as a piano teacher at the music school of Ettiswil, Switzerland
- Working experience (i.e., competences and responsibilities, laboratory techniques, field and teaching experience): see *Annex to CV*

### ***Presentations at Meetings, Congresses:***

- 1991: Wicki A, Marfurt J, Weiss N.  
“Specific and sensitive detection of *Entamoeba histolytica* by the polymerase chain reaction”  
50<sup>th</sup> Annual Meeting of the Swiss Society of Microbiology (SSM), Basel, Switzerland, April 4-6, 1991.
- 2000: Miot S, Marfurt J, Lach E, Sadallah S, Schifferli JA.  
“Generation of an antibody against the intracellular domain of CR 1”  
XVIII<sup>th</sup> International Complement Workshop, Salt Lake City, Utah, USA, July 23-27, 2000.
- 2002: Marfurt J, Niederwieser I, Makia D., Dobler M., Beck H-P., Felger I.  
“Diagnosis and differentiation of *Leishmania* species in clinical samples”  
Annual Congress of the Swiss Society of Tropical Medicine and Parasitology (SSTMP), Bern, October 14-15, 2002.
- 2003: Marfurt J, Felger I., Beck H-P., Genton B.  
“DNA chip technology: a new epidemiological tool to monitor drug resistance in malaria?”  
Joint Annual Meeting of the Swiss Societies of Microbiology (SSM), Infectious Diseases (SSI), and Tropical Medicine and Hygiene (SSTMP), Basel, Switzerland, March 6-7, 2003.
- 2003: Marfurt J, PhD project presentation  
PhD meeting of the Swiss Societies of Microbiology (SSM), Münchenwiler, Switzerland, October 16-17, 2003.
- 2004: Marfurt J, Sie A., Ivivi R., Gomobi M., Müller I., Reeder J., Beck H-P., Genton B.  
“*In vivo* and molecular monitoring of antimalarial drug resistance in Papua New Guinea”  
Joint Meeting of the German Society of Tropical Medicine and International Health (DTG) and the Swiss Society of Tropical Medicine and Parasitology (SSTMP), Würzburg, Germany, September 23-25, 2004.
- 2004: Marfurt J, Cramer A., Maire N., Regös A., Burki R., Felger I., Genton B., Beck H-P.  
“DNA microarray technology: a new tool for the analysis of malaria drug resistance markers”  
Malaria Meeting of the Paul-Ehrlich Society for Chemotherapy (PEG), Berlin, Germany, October 29-30, 2004.
- 2005: Marfurt J, PhD project presentation  
Seminar at the Queensland Institute of Medical Research (QIMR), Research Division: Infectious Diseases and Immunology, Malaria and Scabies Group, Brisbane, Australia, June 9, 2005.
- 2005: Marfurt J, Müller I., Sie A., Oa O., Lorry K., Reeder JC., Beck H-P., Genton B.  
“Monitoring of antimalarial drug resistance in Papua New Guinea”  
41<sup>st</sup> Annual Symposium of The Medical Society of Papua New Guinea, Goroka, PNG, September 5-9, 2005.
- 2005: 1) Marfurt J, Müller I., Reeder JC., Beck H-P., Genton B.  
“*In vivo* and molecular monitoring of antimalarial drug resistance in Papua New Guinea”  
2) Marfurt J, Müller I., Reeder JC., Beck H-P., Genton B.  
“Clinical versus community monitoring of molecular markers for drug resistant malaria”  
XVI<sup>e</sup> International Congress for Tropical Medicine and Malaria, Marseille, France, September 11-15, 2005.
- 2005: Cramer A., Mugittu K., Marfurt J, Regoes A., Maire N., Burki R., Felger I., Beck H-P.  
“Rapid field applicable microarray-based method for monitoring of all single nucleotide polymorphisms associated with resistance to antimalarial drugs”  
4<sup>th</sup> MIM Pan-African Malaria Conference, Yaoundé, Cameroon, November 13-18, 2005.
- 2006: Marfurt J, Müller I., Reeder JC., Beck H-P., Genton B.  
“Drug resistant *P. falciparum* malaria in Papua New Guinea: evaluation of a community-based approach using DNA microarray technology for the surveillance of resistance”  
Joint Meeting of the Royal Society of Tropical Medicine and Hygiene (RSTM&H) and the Swiss Society of Tropical Medicine and Hygiene (SSTMP), Basel, Switzerland, September 22-23, 2006.

***Publications:***

- Pluschke G., Joss A., Marfurt J., Daubenberg C., Kashala O., Zwickl M., Stief A., Sansig G., Schlapfer B., Linkert S., van der Putten H., Hardmann N., Schroeder M.  
Generation of chimeric monoclonal antibodies from mice that carry human immunoglobulin *Cgamma1* heavy of *Ckappa* light chain gene segments.  
*J Immunol Methods*. 1998 Jun 1; 215(1-2):27-37.
- Favre N., Daubenberg C., Marfurt J., Moreno A., Patarroyo M., Pluschke G.  
Sequence and diversity of T-cell receptor *alpha* V, J, and C genes of the owl monkey *Aotus nancymae*.  
*Immunogenetics*. 1998 Sep;48(4):253-9.
- Trendelenburg M, Marfurt J., Gerber I, Tyndall A, Schifferli JA.  
Lack of occurrence of severe lupus nephritis among anti-C1q autoantibody-negative patients.  
*Arthritis Rheum*. 1999 Jan;42(1):187-8.
- Lach-Trifilieff E., Marfurt J., Schwarz S., Sadallah S., Schifferli JA.  
Complement receptor 1 (CD35) on human reticulocytes: normal expression in systemic lupus erythematosus and HIV-infected patients.  
*J Immunol*. 1999 Jun 15;162(12):7549-54.
- Miot S., Marfurt J., Lach-Trifilieff E., Gonzalez-Rubio C., Lopez-Trascasa M., Sadallah S., Schifferli JA.  
The mechanism of loss of CR1 during maturation of erythrocytes is different between Factor I deficient patients and healthy donors.  
*Blood Cells Mol Dis*. 2002 Sep-Oct; 29(2):200-12.
- Marfurt J., Nasereddin A., Niederwieser I., Jaffe CL., Beck H-P., Felger I.  
Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the minixon sequence and subsequent restriction fragment length polymorphism analysis.  
*J Clin Microbiol*. 2003 Jul; 41(7):3147-53.
- Marfurt J., Niederwieser I., Makia ND., Beck H-P., Felger I.  
Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP.  
*Diagn Microbiol Infect Dis*. 2003 Jun; 46(2):115-24.
- Müller I., Tulloch J., Marfurt J., Hide R., Reeder JC. 2006  
Malaria control in Papua New Guinea results in complex epidemiological changes.  
In press (PNG Medical Journal)
- Marfurt J., Müller I., Sie A., Maku P., Goroti M., Reeder JC., Beck H-P., Genton B. 2006  
Low efficacy of amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against falciparum and vivax malaria in Papua New Guinea.  
Submitted
- Cramer A., Marfurt J., Mugittu K., Maire N., Regös A., Coppee J-Y., Sismeiro O., Burki R., Huber E., Laubscher D., Puijalon O., Genton B., Felger I., Beck H-P. 2006.  
A rapid field applicable microarray-based method for monitoring all single nucleotide polymorphisms associated with resistance to antimalarial drugs.  
Submitted
- Marfurt J., Müller I., Smith TA., Reeder JC., Beck H-P., Genton B. 2006  
The usefulness of twenty-four molecular markers in predicting treatment outcome with combination therapy of amodiaquine or chloroquine plus sulphadoxine-pyrimethamine against uncomplicated malaria in Papua New Guinea.  
In preparation
- Marfurt J., de Monbrison F., Brega S., Barbolat L., Müller I., Reeder JC., Beck H-P., Picot S., Genton B. 2006  
Association of mutations in *Plasmodium vivax dhfr* and *mdr1* with *in vivo* resistance to amodiaquine or chloroquine plus sulphadoxine-pyrimethamine in Papua New Guinea.  
In preparation
- Marfurt J., Müller I., Smith TA., Bretscher M., Reeder JC., Beck H-P., Genton B. 2006  
Community versus clinical molecular monitoring of drug resistant malaria.  
In preparation
- Marfurt J., Müller I., Smith TA., Coppee J-Y., Sismeiro O., Reeder JC., Beck H-P., Genton B. 2006  
Drug resistant *P. falciparum* malaria in Papua New Guinea: Evaluation of a community-based approach using DNA microarray technology for the surveillance of resistance.  
In preparation